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<b>(21) International Application Number:</b> PCT/GB96/03186 <b>(22) International Filing Date:</b> 20 December 1996 (20.12.96)  <b>(30) Priority Data:</b> <table border="0"><tr><td>9526083.2</td><td>20 December 1995 (20.12.95)</td><td>GB</td></tr><tr><td>9610272.8</td><td>16 May 1996 (16.05.96)</td><td>GB</td></tr><tr><td>9615066.9</td><td>18 July 1996 (18.07.96)</td><td>GB</td></tr></table> <b>(71) Applicants (for all designated States except US):</b> MEDICAL RESEARCH COUNCIL [GB/GB]; 20 Park Crescent, London W1N 4AL (GB). THE UNIVERSITY OF DUNDEE [GB/GB]; Tower Building, Dundee DD1 4HN (GB).  <b>(72) Inventors; and</b> <b>(75) Inventors/Applicants (for US only):</b> COHEN, Philip [GB/GB]; Inverbay II, Invergowrie, by Dundee, Dundee DD2 5DQ (GB). ALESSI, Dario [GB/GB]; 45 Baldovan Terrace, Dundee DD4 6NJ (GB). CROSS, Darren [GB/GB]; 5 Pitkerro Road, Dundee DD4 7E7 (GB).  <b>(74) Agent:</b> MURGITROYD & COMPANY; 373 Scotland Street, Glasgow G5 8QA (GB).		9526083.2	20 December 1995 (20.12.95)	GB	9610272.8	16 May 1996 (16.05.96)	GB	9615066.9	18 July 1996 (18.07.96)	GB	<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, TJ, TM, TR, TT, UA, UG, US, UZ, VN, ARIPO patent (KE, LS, MW, SD, SZ, UG), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
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<b>(54) Title:</b> CONTROL OF PROTEIN SYNTHESIS, AND SCREENING METHOD FOR AGENTS  <b>(57) Abstract</b>  A method for screening for agents capable of affecting the activity of kinases GSK3 and PKB is disclosed. The method involves assessing the phosphorylation of PKB on two amino acids on the PKB molecule particularly.											

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1     Control of protein synthesis, and screening method for  
2     agents.

3  
4     The present invention relates to the control of  
5     glycogen metabolism and protein synthesis, in  
6     particular through the use of insulin.

7  
8     Many people with diabetes have normal levels of insulin  
9     in their blood, but the insulin fails to stimulate  
10    muscle cells and fat cells in the normal way (type II  
11    diabetes). Currently it is believed that there is a  
12    breakdown in the mechanism through which insulin  
13    signals to the muscle and fat cells.

14  
15    The enzyme glycogen synthase kinase-3 (GSK3) (Embi et  
16    al., 1980) is implicated in the regulation of several  
17    physiological processes, including the control of  
18    glycogen (Parker et al., 1983) and protein (Welsh et  
19    al., 1993) synthesis by insulin, modulation of the  
20    transcription factors AP-1 and CREB (Nikolaki et al, de  
21    Groot et al., 1993 and Fiol et al 1994), the  
22    specification of cell fate in *Drosophila* (Siegfried et  
23    al., 1992) and dorsoventral patterning in *Xenopus*  
24    embryos (He et al., 1995). GSK3 is inhibited by serine

1 phosphorylation in response to insulin or growth  
2 factors (Welsh et al., 1993, Hughes et al., 1994, Cross  
3 et al., 1994 and Saito et al., 1994) and *in vitro* by  
4 either MAP kinase-activated protein (MAPKAP) kinase-1  
5 (also known as p90<sup>rk</sup>) or P70 ribosomal S6 kinase (p70<sup>S6k</sup>)  
6 (Sutherland et al., 1993 and Sutherland et al., 1994).

7  
8 We have now found, however, that agents which prevent  
9 the activation of both MAPKAP kinase-1 and p70<sup>S6k</sup> by  
10 insulin *in vivo* do not block the phosphorylation and  
11 inhibition of GSK3. Another insulin-stimulated protein  
12 kinase inactivates GSK3 under these conditions, and we  
13 demonstrate that it is the product of the proto-  
14 oncogene Akt (also known as RAC or PKB; herein referred  
15 to as "PKB").

16  
17 GSK3 is inhibited in response to insulin with a half  
18 time of two min, slightly slower than the half time for  
19 activation of PKB $\alpha$  (one min). Inhibition of GSK3 by  
20 insulin results in its phosphorylation at the same  
21 serine residue (serine 21) which is targeted by PKB $\alpha$  *in*  
22 *vitro*. Like the activation of PKB $\alpha$ , the inhibition of  
23 GSK3 by insulin is prevented by phosphatidyl inositol  
24 (PI-3) kinase inhibitors wortmannin and LY 294002. The  
25 inhibition of GSK3 is likely to contribute to the  
26 increase in the rate of glycogen synthesis (Cross et  
27 al., 1994) and translation of certain mRNAs by insulin  
28 (Welsh et al., 1994).

29  
30 Two isoforms of PKB, termed PKB $\alpha$  (Coffer & Woodgett,  
31 1991), PKB $\beta$  (Cheng et al., 1992) and PKB $\gamma$  (Konishi et  
32 al., 1995) have been identified and characterised.  
33 PKB $\beta$ , also known as RAC $\beta$  and Akt-2, is over-expressed  
34 in a significant number of ovarian (Cheng et al., 1992)  
35 and pancreatic (Cheng et al., 1996) cancers and is  
36 over-expressed in the breast cancer epithelial cell

1 line MCF7. PKB is composed of an N-terminal pleckstrin  
2 homology (PH) domain, followed by a catalytic domain  
3 and a short C-terminal tail. The catalytic domain is  
4 most similar to cyclic AMP-dependent protein kinase  
5 (PKA, 65% similarity) and to protein kinase C (PKC, 75%  
6 similarity) findings that gave rise to two of its  
7 names, namely PKB (i.e. between PKA and PKC) and RAC  
8 (Related to A and C kinase).

9  
10 Many growth factors trigger the activation of  
11 phosphatidylinositol (PI) 3-kinase, the enzyme which  
12 converts PI 4,5 bisphosphate (PIP2) to the putative  
13 second messenger PI 3,4,5 trisphosphate (PIP3), and PKB  
14 lies downstream of PI 3-kinase (Franke et al., 1995).  
15 PKB $\alpha$  is converted from an inactive to an active form  
16 with a half time of about one minute when cells are  
17 stimulated with PDGF (Franke et al., 1995), EGF or  
18 basic FGF (Burgering & Coffey, 1995) or insulin (Cross  
19 et al., 1995 and Kohn et al., 1995) or perpervanadate  
20 (Andjelkovic et al., 1996). Activation of PKB by  
21 insulin or growth factors is prevented if the cells are  
22 preincubated with inhibitors of PI 3-kinase (wortmannin  
23 or LY 294002) or by overexpression of a dominant  
24 negative mutant of PI 3-kinase (Burgering & Coffey  
25 1995). Mutation of the tyrosine residues in the PDGF  
26 receptor that when phosphorylated bind to PI 3-kinase  
27 also prevent the activation of PKB $\alpha$  (Burgering &  
28 Coffey, 1995 and Franke et al., 1995).

29  
30 The present invention thus provides the use of PKB, its  
31 analogues, isoforms, inhibitors, activators and/or the  
32 functional equivalents thereof to regulate glycogen  
33 metabolism and/or protein synthesis, in particular in  
34 disease states where glycogen metabolism and/or protein  
35 synthesis exhibits abnormality, for example in the  
36 treatment of type II diabetes; also in the treatment of

1 cancer, such as ovarian, breast and pancreatic cancer.  
2 A composition comprising such agents is also covered by  
3 the present invention, and the use of such a  
4 composition for treatment of disease states where  
5 glycogen metabolism and/or protein synthesis exhibit  
6 abnormality.

7  
8 The present invention also provides a novel peptide  
9 comprising the amino acid sequence Arg-Xaa-Arg-Yaa-Zaa-  
10 Ser/Thr-Hyd, where Xaa is any amino acid, Yaa and Zaa  
11 are any amino acid (preferably not glycine), and Hyd is  
12 a large hydrophobic residue such as Phe or Leu, or a  
13 functional equivalent thereof. Represented in single  
14 letter code, a suitable peptide would be RXXR'X'S/TF/L,  
15 where X' can be any amino acid, but is preferably not  
16 glycine; glycine can in fact be used, but other amino  
17 acids are preferred. Typical peptides include  
18 GRPRTSSFAEG, RPRAATC or functional equivalents thereof.  
19 The peptide is a substrate for measuring PKB activity.

20  
21 The invention also provides a method for screening for  
22 substances which inhibit the activation of PKB in vivo  
23 by preventing its interaction with PIP3 or PI3,4-bisP.

24  
25 Thus the invention also provides a method of  
26 determining the ability of a substance to affect the  
27 activity or activation of PKB, the method comprising  
28 exposing the substance to PKB and phosphatidyl inositol  
29 polyphosphate (ie PIP3, PI3,4-bisP etc) and determining  
30 the interaction between PKB and the phosphatidyl  
31 inositol polyphosphate. The interaction between PKB  
32 and the phosphatidyl inositol polyphosphate can  
33 conveniently be measured by assessing the  
34 phosphorylation state of PKB (preferably at T308 and/or  
35 S473), eg by measuring transfer of radiolabelled <sup>32</sup>P  
36 from the PIP3 (for example) to the PKB and/or by SDS-

1 PAGE.

2  
3 The method of the invention can also be used for  
4 identifying activators or inhibitors of GSK3, such a  
5 method can comprise exposing the substance to be tested  
6 to GSK3, and (optionally) a source of phosphorylation,  
7 and determining the state of activation of GSK3  
8 (optionally by determining the state of its  
9 phosphorylation. This aspect of the invention can be  
10 useful for determining the suitability of a test  
11 substance for use in combatting diabetes, cancer, or  
12 any disorder which involves irregularity of protein  
13 synthesis or glycogen metabolism.

14  
15 The invention also provides a method for screening for  
16 inhibitors or activators of enzymes that catalyse the  
17 phosphorylation of PKB, the method comprising exposing  
18 the substance to be tested to  
19 - one or more enzymes upstream of PKB;  
20 - PKB; and (optionally)  
21 - nucleoside triphosphate  
22 and determining whether (and optionally to what extent)  
23 the PKB has been phosphorylated on T308 and/or S473.

24  
25 Also provided is a method of identifying agents able to  
26 influence the activity of GSK3, said method comprising:

- 27  
28 a. exposing a test substance to a substrate of GSK3;  
29  
30 b. detecting whether (and, optionally, to what  
31 extent) said peptide has been phosphorylated.

32  
33 The test substance may be an analogue, isoform,  
34 inhibitor, or activator of PKB, and the above method  
35 may be modified to identify those agents which  
36 stimulate or inhibit PKB itself. Thus such a method

1 may comprise the following steps:

2

3 a. exposing the test substance to a sample containing  
4 PKB, to form a mixture;

5

6 b. exposing said mixture to a peptide comprising the  
7 amino acid sequence defined above or a functional  
8 equivalent thereof (usually in the presence of  $Mg^{2+}$   
9 and ATP); and

10

11 c. detecting whether (and, optionally, to what  
12 extent) said peptide has been phosphorylated.

13

14 In this aspect the method of the invention can be used  
15 to determine whether the substance being tested acts on  
16 PKB or directly on GSK3. This can be done by comparing  
17 the phosphorylation states of the peptide and PKB; if  
18 the phosphorylation state of GSK3 is changed but that  
19 of PKB is not then the substance being tested acts  
20 directly on GSK3 without acting on PKB.

21 In a further aspect the present invention provides a  
22 method of treatment of the human or non-human  
23 (preferably mammalian) animal body, said method  
24 comprising administering PKB, its analogues,  
25 inhibitors, stimulators or functional equivalents  
26 thereof to said body. Said method affects the  
27 regulation of glycogen metabolism in the treated body.

28

29 The method of treatment of the present invention may be  
30 of particular use in the treatment of type II diabetes  
31 (where desirably an activator of PKB is used, so that  
32 the down-regulation of GSK3 activity due to the action  
33 of PKB is enhanced).

34

35 The method of treatment of the present invention may  
36 alternatively be of particular use in the treatment of



1 cancer such as ovarian cancer (where desirably an  
2 inhibitor of PKB is used, so that the down-regulation  
3 of GSK3 activity due to the action of PKB is  
4 depressed). Other cancers associated with  
5 irregularities in the activity of PKB and/or GSK3 may  
6 also be treated by the method, such as pancreatic  
7 cancer, and breast cancer.

8  
9 Stimulation of PKB with insulin increases activity  
10 12-fold within 5 min and induces its phosphorylation at  
11 Thr-308 and Ser-473. PKB transiently transfected into  
12 cells can be activated 20-fold in response to insulin  
13 and 46-fold in response to IGF-1 and also became  
14 phosphorylated at Thr-308 and Ser-473. The activation  
15 of PKB and its phosphorylation at both Thr-308 and  
16 Ser-473 can be prevented by the phosphatidylinositol  
17 (PI) 3-kinase inhibitor wortmannin. The  
18 phosphorylation of threonine 308 and serine 473 act  
19 synergistically to activate PKB.

20  
21 MAPKAP kinase-2-phosphorylated PKB at Ser-473 in vitro  
22 increases activity seven-fold, an effect that can be  
23 mimicked (fivefold activation) by mutating Ser-473 to  
24 Asp. Mutation of Thr-308 to Asp also increases PKB  
25 activity five-fold and subsequent phosphorylation of  
26 Ser-473 by MAPKAP kinase-2 stimulates activity a  
27 further fivefold, an effect mimicked (18-fold  
28 activation) by mutating both Thr-308 and Ser-473 to  
29 Asp. The activity of the Asp-308/Asp-473 double mutant  
30 was similar to that of the fully phosphorylated enzyme  
31 and could not be activated further by insulin. Mutation  
32 of Thr-308 to Ala did not prevent the phosphorylation  
33 of transfected PKB at Ser-473 after stimulation of 293  
34 cells with insulin or IGF-1, but abolished the  
35 activation of PKB. Similarly, mutation of Ser-473 to  
36 Ala did not prevent the phosphorylation of transfected

1     PKB at Thr-308 but greatly reduced the activation of  
2     transfected PKB. This demonstrates that the activation  
3     of PKB by insulin or IGF-1 results from the  
4     phosphorylation of Thr-308 and Ser-473 and that  
5     phosphorylation of both residues is preferred to  
6     generate a high level of PKB activity in vitro or in  
7     vivo. Also, phosphorylation of Thr-308 in vivo is not  
8     dependent on the phosphorylation of Ser-473 or vice  
9     versa, that the phosphorylation of Thr-308 and Ser-473  
10    are both dependent on PI 3-kinase activity and suggest  
11    that neither Thr-308 nor Ser-473 phosphorylation is  
12    catalysed by PKB itself.

13  
14    Thus, it is preferred that the present invention  
15    incorporates the use of any agent which affects  
16    phosphorylation of PKB at amino acids 308 and/or 473,  
17    for example insulin, inhibitors of PI 3-kinase such as  
18    wortmannin or the like. The use of PKB, itself altered  
19    at amino acids 308 and/or 473 (eg by phosphorylation  
20    and/or mutation) is also suitable.

21  
22    In a variation of the method of the present invention,  
23    stimulation or inhibition of PKB may be assessed by  
24    monitoring the phosphorylation states of amino acids  
25    308 and/or 473 on PKB itself.

26  
27    Different isoforms of PKB may be used or targeted in  
28    the present invention; for example PKB $\alpha$ ,  $\beta$  or  $\gamma$ .

29  
30    The present invention will now be described in more  
31    detail in the accompanying examples which are provided  
32    by way of non-limiting illustration, and with reference  
33    to the accompanying drawings.

34

35    Example 1: PKB influences GSK3 activity.

36    Fig 1: a, L6 myotubes were incubated for 15 min with 2

1 mM 8-bromocyclic-AMP (8Br-cAMP) and then with 0.1  $\mu$ M  
2 insulin (5 min). Both GSK3 isoforms were co-  
3 immunoprecipitated from the lysates and assayed before  
4 (black bars) and after (white bars) reactivation with  
5 PP2A (Cross et al., 1994). The results are presented  
6 relative to the activity in unstimulated cells, which  
7 was  $0.08 \pm 0.006$  U  $\text{mg}^{-1}$  ( $n=10$ ).  
8 *b, c*, The inhibition of GSK3 by insulin (0.1  $\mu$ M) is  
9 unaffected by rapamycin (0.1  $\mu$ M) and PD 98059 (50  $\mu$ M),  
10 but prevented by LY 294002 (100  $\mu$ M).  
11  
12 *b*, L6 myotubes were stimulated with insulin for the  
13 times indicated with (filled triangle) or without  
14 (filled circles) a 15 min preincubation with LY 294002,  
15 and GSK3 measured as in *a*. The open circles show  
16 experiments from insulin-stimulated cells where GSK3  
17 was assayed after reactivation with PP2A (Cross et al.,  
18 1994).  
19  
20 *c*, cells were incubated with rapamycin (triangles) or  
21 rapamycin plus PD 98059 (circles) before stimulation  
22 with insulin, and GSK3 activity measured as in *a*,  
23 before (filled symbols) and after (open symbols)  
24 pretreatment with PP2A.  
25  
26 *d, e*, L6 myotubes were incubated with 8Br-cAMP (15 min)  
27 PD 98059 (60 min) or LY 294002 (15 min) and then with  
28 insulin (5 min) as in *a-c*. Each enzyme was assayed  
29 after immunoprecipitation from lysates, and the results  
30 are presented relative to the activities obtained. In  
31 the presence of insulin and absence of 8Br-cAMP, which  
32 were  $0.04 \pm 0.005$  U  $\text{mg}^{-1}$  (p42 MAP kinase,  $n=6$ ) and  $0.071 \pm$   
33  $0.004$  U  $\text{mg}^{-1}$  (MAPKAP Kinase<sup>-1</sup>,  $n=6$ ).  
34  
35 All the results ( $\pm$  s.e.m.) are for at least three  
36 experiments.

1 Monolayers of L6 cells were cultured, stimulated and  
2 lysed as described previously (Cross et al., 1994).  
3 p42 MAP kinase, MAPKAP kinase 1 or (GSK3- $\alpha$  plus GSK3- $\beta$ )  
4 were then immunoprecipitated from the lysates and  
5 assayed with specific protein or peptide substrates as  
6 described previously (Cross et al., 1994). One unit of  
7 protein kinase activity was that amount which catalysed  
8 the phosphorylation of 1 nmol of substrate in 1 min.  
9 Where indicated, GSK3 in immunoprecipitates was  
10 reactivated with PP2A (Cross et al., 1994).

11

12

13 Figure 2 Identification of PKB as the insulin-  
14 stimulated, wortmannin-sensitive and PD  
15 98059/rapamycin-insensitive Crosstide kinase in L6  
16 myotubes.

17 a. Cells were incubated with 50  $\mu$ M PD 98059 (for 1  
18 hour) and 0.1  $\mu$ M rapamycin (10 min), then stimulated  
19 with 0.1  $\mu$ M insulin (5 min) and lysed (Cross et al.,  
20 1994). The lysates (0.3 mg protein) were  
21 chromatographed on Mono Q (5 x 0.16cm) and fractions  
22 (0.05ml) were assayed for Crosstide kinase (filled  
23 circles). In separate experiments insulin was omitted  
24 (open circles) or wortmannin (0.1  $\mu$ M) added 10 min  
25 before the insulin (filled triangles). The broken line  
26 shows the NaCl gradient. Similar results were obtained  
27 in six experiments.

28

29 b. Pooled fractions (10  $\mu$ l) 31-34 (lane 1), 35-38 (lane  
30 2), 39-42 (lane 3), 43-45 (lane 4), 46-49 (lane 5) and  
31 50-53 (lane 6) from a were electrophoresed on a 10%  
32 SDS/polyacrylamide gel and immunoblotted with the C-  
33 terminal anti-PKB $\alpha$  antibody. Marker proteins are  
34 indicated. No immunoreactive species were present in  
35 fractions 1-30 or 54-80.

36

1 c. L6 myotubes were stimulated with 0.1  $\mu$ M insulin and  
2 PKB immunoprecipitated from the lysates (50  $\mu$ g protein)  
3 essentially as described previously (Lazar et al.,  
4 1995), using the anti-PH domain antibody and assayed  
5 for Crosstide kinase (open circles). In control  
6 experiments, myotubes were incubated with 0.1  $\mu$ M  
7 rapamycin plus 50  $\mu$ M PD 98059 (open triangles) or 2 mM  
8 8Br-cAMP (open squares), or 0.1  $\mu$ M wortmannin (filled  
9 circles) or 100  $\mu$ M LY 294002 (filled triangles) before  
10 stimulation with insulin.

11  
12 d. As c, except that MAPKAP kinase-1 was  
13 immunoprecipitated from the lysates and assayed with S6  
14 peptide (filled circles). In control experiments,  
15 cells were incubated with 0.1  $\mu$ M rapamycin plus 50  $\mu$ M  
16 PD 98059 (filled triangles) or with 2  $\mu$ M 8Br-cAMP (open  
17 circles) before stimulation with insulin. In c and d,  
18 the error bars denote triplicate determinations, and  
19 similar results were obtained in three separate  
20 experiments.

21  
22 Mono Q chromatography was performed as described  
23 (Burgering et al., 1995), except that the buffer also  
24 contained 1 mM EGTA, 0.1 mM sodium orthovanadate and  
25 0.5% (w/v) Triton X-100. Two PKB $\alpha$  antibodies were  
26 raised in rabbits against the C-terminal peptide  
27 FPQFSYSASSTA and bacterially expressed PH domain of  
28 PKB $\alpha$ . The C-terminal antibody was affinity purified  
29 (Jones et al., 1991). The activity of PKB towards  
30 Crosstide is threefold higher than its activity towards  
31 histone H2B and 11-fold higher than its activity  
32 towards myelin basic protein, the substrates used  
33 previously to assay PKB. Other experimental details  
34 and units of protein kinase activity are given in  
35 Fig 1.

36

1 Figure 3 GSK3 is inactivated by PKB from insulin-  
2 stimulated L6 myotubes.

3 a. Cells were stimulated for 5 min with 0.1  $\mu$ M insulin,  
4 and PKB immunoprecipitated from 100  $\mu$ g of cell lysate  
5 and used to inactivate GSK3 isoforms essentially as  
6 described previously (Sutherland et al., 1993 and  
7 Sutherland et al., 1994). The black bars show GSK3  
8 activity measured after incubation with MgATP and PKB  
9 as a percentage of the activity obtained in control  
10 incubations where PKB was omitted. In the absence of  
11 PKB, GSK3 activity was stable throughout the  
12 experiment. The white bars show the activity obtained  
13 after reactivation of GSK3 with PP2A (Embi et al.,  
14 1980). No inactivation of GSK3 occurred if insulin was  
15 omitted, or if wortmannin (0.1  $\mu$ M) was added 10 min  
16 before the insulin, or if the anti-PKB antibody was  
17 incubated with peptide immunogen (0.5 mM) before  
18 immunoprecipitation. The results ( $\pm$  s.e.m.) are for  
19 three experiments (each carried out in triplicate).

20  
21 b. Inactivation of GSK3- $\beta$  by HA-PKB $\alpha$ . Complementary  
22 DNA encoding HA-PKB $\alpha$  was transfected into COS-1 cells,  
23 and after stimulation for 15 min with 0.1 mM sodium  
24 pervanadate the tagged protein kinase was  
25 immunoprecipitated from 0.3 mg of lysate and incubated  
26 for 20 min with GSK3- $\beta$  and MgATP. In control  
27 experiments, pervanadate was omitted, or wildtype (WT)  
28 PKB $\alpha$  replaced by vector (mock translation) or by a  
29 kinase-inactive mutant of PKB $\alpha$  in which Lys 179 was  
30 mutated to Ala (K179A). Similar results were obtained  
31 in three separate experiments. The levels of WT and  
32 K179A-PKB $\alpha$  in each immunoprecipitate were similar in  
33 each transfection.

34  
35 In a GSK3- $\alpha$  and GSK3- $\beta$  were partially purified,  
36 assayed, inactivated by PKB, and reactivated by PP2A

1 from rabbit skeletal muscle as described previously  
2 (Sutherland et al., 1993 and Sutherland et al.,  
3 1994). There was no reactivation in control  
4 experiments in which okadaic acid (2  $\mu$ M) was added  
5 before PP2A.

6

7 Figure 4: Identification of the residues in GSK3  
8 phosphorylated by PKB in vitro and in response to  
9 insulin in L6 myotubes.

10 a. GSK3- $\beta$  was maximally inactivated by incubation with  
11 PKB and Mg-[ $\gamma$ - $^{32}$ P]ATP and after SDS-PAGE, the  $^{32}$ P-  
12 labelled GSK3- $\beta$  (M, 47K) was digested with trypsin<sup>11</sup> and  
13 chromatographed on a C<sub>18</sub> column (Sutherland et al.,  
14 1993). Fractions (0.8 ml) were analysed for  $^{32}$ P-  
15 radioactivity (open circles), and the diagonal line  
16 shows the acetonitrile gradient.

17

18 b. The major phosphopeptide from a (400 c.p.m.) was  
19 subjected to solid-phase sequencing (Sutherland et al.,  
20 1993), and  $^{32}$ P-radioactivity released after each cycle  
21 of Edman degradation is shown.

22

23 c. GSK3- $\alpha$  and GSK3- $\beta$  were co-immunoprecipitated from  
24 the lysates of  $^{32}$ P-labelled cells, denatured in SDS,  
25 subjected to SDS-PAGE, transferred to nitrocellulose  
26 and autoradiographed (Saito et al., 1994). Lanes 1-3,  
27 GSK3 isoforms immunoprecipitated from unstimulated  
28 cells; lanes 4-6, GSK3 isoforms immunoprecipitated from  
29 insulin-stimulated cells.

30

31 d. GSK3 isoforms from c. were digested with trypsin,  
32 and the resulting phosphopeptides separated by  
33 isoelectric focusing (Saito et al., 1994) and  
34 identified by auto-radiography. Lanes 1 and 4 show the  
35 major phosphopeptide resulting from in vitro  
36 phosphorylation of GSK3- $\beta$  by PKB and MAPKAP kinase-1,

1        respectively; lanes 2 and 5, the phosphopeptides  
2        obtained from GSK3- $\beta$  and GSK3- $\alpha$ , immunoprecipitated  
3        from unstimulated cells; lanes 3 and 6, the  
4        phosphopeptides obtained from GSK3- $\beta$  and GSK3- $\alpha$   
5        immunoprecipitated from cells stimulated for 5 min with  
6        0.1  $\mu$ M insulin; the arrow denotes the peptides whose  
7        phosphorylation is increased by insulin. The pI values  
8        of two markers, Patent Blue (2.4) and azurin (5.7) are  
9        indicated.

10

11       In a. PKB $\alpha$  was immunoprecipitated with the C-terminal  
12       antibody from the lysates (0.5 mg protein) of insulin-  
13       stimulated L6 myotubes and used to phosphorylate GSK-  
14        $\beta^{12}$ . In c. three 10-cm diameter dishes of L6 myotubes  
15       were incubated for 4 hours in HEPES-buffered saline  
16       (Cross et al., 1994) containing 50  $\mu$ M PD 98059, 100 nM  
17       rapamycin and 1.5 mCi ml<sup>-1</sup> <sup>32</sup>P-orthophosphate, stimulated  
18       for 5 min with insulin (0.1  $\mu$ M) or buffer, and GSK3  
19       isoforms co-immunoprecipitated from the lysates as in  
20       Fig 1.

21

## 22       Discussion.

23       Inhibition of GSK3 induced by insulin in L6 myotubes  
24       (Fig 1a-c) was unaffected by agents which prevented the  
25       activation of MAPKAP kinase-1 (8-bromo-cyclic AMP, or  
26       PD 98059 (Alessi et al., 1995), (Fig 1d,e) and/or p70<sup>S6k</sup>  
27       (rapamycin (Kuo et al., 1992)) (Cross et al., 1994),  
28       suggesting that neither MAPKAP kinase-1 nor p70<sup>S6k</sup> are  
29       essential for this process. However, the  
30       phosphorylation and inhibition of GSK3- $\beta$  after phorbol  
31       ester treatment (Stambolic et al., 1994) is enhanced by  
32       coexpression with MAPKAP kinase 1 in HeLa S3 cells,  
33       whereas in NIH 3T3 cells the EGF-induced inhibition of  
34       GSK3- $\alpha$  and GSK3- $\beta$  (Saito et al., 1994) is largely  
35       suppressed by expression of a dominant-negative mutant  
36       of MAP kinase kinase-1 (Elgar et al., 1995). MAPKAP



1 kinase-1 may therefore mediate the inhibition of GSK3  
2 by agonists which are much more potent activators of  
3 the classical MAP kinase pathway than is insulin.

4  
5 To identify the insulin-stimulated protein kinase that  
6 inhibits GSK3 in the presence of rapamycin and PD  
7 98059, L6 myotubes were incubated with both compounds  
8 and stimulated with insulin. The lysates were then  
9 chromatographed on Mono Q and the fractions assayed  
10 with "Crosstide" (GRPRTSSFAEG), a peptide corresponding  
11 to the sequence in GSK3 surrounding the serine  
12 (underlined) phosphorylated by MAPKAP kinase-1 and p70<sup>S6k</sup>  
13 (Ser 21 in GSK3- $\alpha$  (Sutherland et al., 1994) and Ser 9  
14 in GSK3- $\beta$  (Sutherland et al 1993)). Three peaks of  
15 Crosstide kinase activity were detected, which were  
16 absent if insulin stimulation was omitted or if the  
17 cells were first preincubated with the PI 3-kinase  
18 inhibitor wortmannin (Fig 2a). Wortmannin (Cross et  
19 al., 1994 and Welsh et al 1994), and the structurally  
20 unrelated PI 3-kinase inhibitor LY 294002 (ref 19);  
21 (Fig 1b), both prevent the inhibition of GSK3 by  
22 insulin.

23  
24 The protein kinases PKB- $\alpha$ , PKB- $\beta$  and PKB $\gamma$  are Ser/Thr-  
25 specific and cellular homologues of the viral oncogene  
26 v-akt (Coffer et al., 1991, Jones et al 1991, Ahmed et  
27 al 1995 and Cheng et al., 1992). These enzymes have  
28 recently been shown to be activated in NIH 3T3, Rat-1  
29 or Swiss 3T3 cells in response to growth factors or  
30 insulin, activation being suppressed by blocking the  
31 activation of PI 3-kinase in different ways (Franke et  
32 al., 1995 and Burgering et al., 1995). All three peaks  
33 of Crosstide kinase (Fig 2a), but no other fraction  
34 from Mono Q, showed the characteristic multiple bands  
35 of PKB (relative molecular mass, M, 58K, 59K or 60K)  
36 that have been observed in other cells, when

1 immunoblotting was performed with an antibody raised  
2 against the carboxyl-terminal peptide of PKB- $\alpha$  (Fig  
3 2b). The more slowly migrating forms represent more  
4 highly phosphorylated protein, and are converted to the  
5 fastest migrating species by treatment with  
6 phosphatases. Phosphatase treatment also results in  
7 the inactivation of PKB (Burgering et al., 1995) and  
8 the complete loss of Crosstide kinase activity (data  
9 not shown). Of the Crosstide kinase activity in peaks  
10 2 and 3 from Mono Q, 70-80% was immunoprecipitated by a  
11 separate antibody raised against the amino-terminal  
12 pleckstrin homology (PH) domain of PKB- $\alpha$ . The C-  
13 terminal antibody also immunoprecipitated PKB activity  
14 specifically from peaks 2 and 3, but was less effective  
15 than the anti-PH-domain antibody. Peak-1 was hardly  
16 immunoprecipitated by either antibody and may represent  
17 PKB- $\beta$ . An immunoprecipitating anti-MAPKAP kinase-1  
18 antibody (Cross et al., 1994) failed to deplete any of  
19 the Crosstide kinase activity associated with peaks 1,  
20 2 or 3.

21  
22 Insulin stimulation of L6 myotubes increased PKB  
23 activity by more than tenfold (Fig 2c), and activation  
24 was blocked by wortmannin or LY 294002, but was  
25 essentially unaffected by 8-bromo-cyclic AMP or  
26 rapamycin plus PD 98059 (Fig 2c). The half-time ( $t_{0.5}$ )  
27 or activation of PKB (1 min) was slightly faster than  
28 that for inhibition of GSK3 (2 min) (Cross et al.,  
29 1994). In contrast, the activation of MAPKAP kinase-1  
30 (Fig 2d) and p70<sup>S6k</sup> (not shown) was slower ( $t_{0.5} > 5$  min).  
31 Activation of MAPKAP kinase-1 was prevented by 8-bromo-  
32 cyclic AMP or PD 98059 (Fig 2d), and activation of p70<sup>S6k</sup>  
33 by rapamycin (Cross et al., 1994). Akt/RAC  
34 phosphorylated the Ser in the Crosstide equivalent to  
35 Ser 21 in GSK3- $\alpha$  and Ser 9 in GSK3- $\beta$  (data not shown).

36

1     PKB from insulin-stimulated L6 myotubes (but not from  
2     unstimulated or wortmannin-treated cells) inactivated  
3     GSK3- $\alpha$  and GSK3- $\beta$  *in vitro*, and inhibition was reversed  
4     by the Ser/Thr-specific protein phosphatase PP2A (Embi  
5     et al., 1980) (Fig 3a). To further establish that  
6     inactivation was catalysed by PKB, and not by a co-  
7     immunoprecipitating protein kinase, haemagglutinin-  
8     tagged PKB- $\alpha$  (HA-PKB) was transfected into COS-1 cells  
9     and activated by stimulation with pervanadate, which is  
10    the strongest inducer of PKB activation in this system.  
11    The HA-PKB inactivated GSK3- $\beta$ , but not if treatment  
12    with pervanadate was omitted or if wild-type HA-PKB was  
13    replaced with a "kinase inactive" mutant (Fig 3b).

14  
15    The inactivation of GSK3- $\beta$  by PKB *in vitro* was  
16    accompanied by the phosphorylation of one major tryptic  
17    peptide (Fig 4a) which coeluted during  $C_{18}$   
18    chromatography (Sutherland et al., 1993) and  
19    isoelectric focusing with that obtained after  
20    phosphorylation by MAPKAP kinase-1 (Fig 4d).  
21    Stimulation of L6 myotubes with insulin (in the  
22    presence of rapamycin and PD 98059) increased the  $^{32}\text{P}$ -  
23    labelling of GSK3- $\alpha$  and GSK3- $\beta$  by 60-100% (Fig 4c) and  
24    increased the  $^{32}\text{P}$ -labelling of the same tryptic peptides  
25    labelled *in vitro* (Fig 4d). Sequence analyses  
26    established that the third residue of these,  
27    corresponding to Ser 9 (GSK3- $\beta$ ) or Ser 21 (GSK3- $\alpha$ ), was  
28    the site of phosphorylation in each phosphopeptide,  
29    both *in vitro* (Fig 4b) and *in vivo* (not shown). The  
30     $^{32}\text{P}$ -labelling of other (more acidic) tryptic  
31    phosphopeptides was not increased by insulin (Fig 4d).  
32    These peptides have been noted previously in GSK3 from  
33    A431 cells and shown to contain phosphoserine and  
34    phosphotyrosine (Saito et al., 1994).

35

36    PKC- $\delta$ ,  $\epsilon$  and  $\zeta$  are reported to be activated by

1 mitogens, and PKC- $\zeta$  activity is stimulated *in vitro* by  
2 several inositol phospholipids, including PI(3,4,5)P<sub>3</sub>,  
3 the product of the PI 3-kinase reaction (Andjelkovic et  
4 al., 1995). However, purified PKC- $\epsilon$  (Palmer et al.,  
5 1995), PKC- $\delta$  and PKC- $\zeta$  (data not shown) all failed to  
6 inhibit GSK3- $\alpha$  or GSK3- $\beta$  *in vitro*. Moreover, although  
7 PKC- $\alpha$ ,  $\beta$ 1 and  $\gamma$  inhibit GSK3- $\beta$  *in vitro* (Palmer et al.,  
8 1995), GSK3- $\alpha$  is unaffected, while their downregulation  
9 in L6 myotubes by prolonged incubation with phorbol  
10 esters abolishes the activation of MAPKAP kinase-1 in  
11 response to subsequent challenge with phorbol esters,  
12 but has no effect on the inhibition of GSK3 by insulin  
13 (not shown).

14

15 Taken together, our results identify GSK3 as a  
16 substrate for PKB. The stimulation of glycogen  
17 synthesis by insulin in skeletal muscle involves the  
18 dephosphorylation of Ser residues in glycogen synthase  
19 that are phosphorylated by GSK3 *in vitro* (Parker et  
20 al., 1983). Hence the 40-50% inhibition of GSK3 by  
21 insulin, coupled with a similar activation of the  
22 relevant glycogen synthase phosphatase (Goode et al.,  
23 1992), can account for the stimulation of glycogen  
24 synthase by insulin in skeletal muscle (Parker et al.,  
25 1983) or L6 myotubes (Goode et al., 1992). The  
26 activation of glycogen synthase and the resulting  
27 stimulation of glycogen synthesis by insulin in L6  
28 myotubes is blocked by wortmannin, but not by PD 98059  
29 (Dent et al., 1990), just like the activation of  
30 Akt/RAC and inhibition of GSK3. However, GSK3 is  
31 unlikely to be the only substrate of PKB *in vivo*, and  
32 identifying other physiologically relevant substrates  
33 will be important because PKB $\beta$  is amplified and over-  
34 expressed in many ovarian neoplasms (Cheng et al.,  
35 1992).

36

1     **Example 2: Activation of PKB by insulin in L6 myotubes**  
2     **is accompanied by phosphorylation of residues Thr-308**  
3     **and Ser-473. Insulin induces the activation and**  
4     **phosphorylation of PKB $\alpha$  in L6 myotubes. Three 10 cm**  
5     **dishes of L6 myotubes were  $^{32}$ P-labelled and treated for**  
6     **10 min with or without 100 nM wortmannin and then for 5**  
7     **min with or without 100 nM insulin. PKB $\alpha$  was**  
8     **immunoprecipitated from the lysates and an aliquot**  
9     **(15%) assayed for PKB $\alpha$  activity (Fig 5A). The**  
10    **activities are plotted  $\pm$  SEM for 3 experiments relative**  
11    **to PKB $\alpha$  derived from unstimulated cells which was 10**  
12    **mU/mg. The remaining 85% of the immunoprecipitated PKB $\alpha$**   
13    **was alkylated with 4-vinylpyridine, electrophoresed on**  
14    **a 10% polyacrylamide gel (prepared without SDS to**  
15    **enhance the phosphorylation-induced decrease in**  
16    **mobility) and autoradiographed. The positions of the**  
17    **molecular mass markers glycogen phosphorylase (97 kDa),**  
18    **bovine serum albumin (66 kDa) and ovalbumin (43 kDa)**  
19    **are marked.**

20  
21    Under these conditions, insulin stimulation resulted in  
22    a 12-fold activation of PKB $\alpha$  (Fig 5A) and was  
23    accompanied by a  $1.9 \pm 0.3$ -fold increase in  
24     $^{32}$ P-labelling (4 experiments) and retardation of its  
25    mobility on SDS-polyacrylamide gels (Fig 5B). The  
26    activation of PKB $\alpha$ , the increase in its  $^{32}$ P-labelling  
27    and reduction in electrophoretic migration were all  
28    abolished by prior incubation of the cells with 100 nM  
29    wortmannin. Phosphoamino acid analysis of the whole  
30    protein revealed that  $^{32}$ P-labelled PKB $\alpha$  was  
31    phosphorylated at both serine and threonine residues  
32    and that stimulation with insulin increased both the  
33     $^{32}$ P-labelling of both phosphoamino acids (data not  
34    shown).

35

36    **Fig. 6. Insulin stimulation of L6 myotubes induces the**

1 phosphorylation of two peptides in PKB $\alpha$ . Bands  
2 corresponding to  $^{32}\text{P}$ -labelled PKB $\alpha$  from Fig 5B were  
3 excised from the gel, treated with 4-vinylpyridine to  
4 alkylate cysteine residues, digested with trypsin and  
5 chromatographed on a Vydac 218TP54 C18 column  
6 (Separations Group, Hesperia, CA) equilibrated with  
7 0.1% (by vol) trifluoroacetic acid (TFA), and the  
8 columns developed with a linear acetonitrile gradient  
9 (diagonal line). The flow rate was 0.8 ml / min and  
10 fractions of 0.4 ml were collected (A) tryptic peptide  
11 map of  $^{32}\text{P}$ -labelled PKB $\alpha$  from unstimulated L6 myotubes;  
12 (B) tryptic peptide map of  $^{32}\text{P}$ -labelled PKB $\alpha$  from  
13 insulin-stimulated L6 myotubes; (C) tryptic peptide map  
14 of  $^{32}\text{P}$ -labelled PKB $\alpha$  from L6 myotubes treated with  
15 wortmannin prior to insulin. The two major  $^{32}\text{P}$ -labelled  
16 peptides eluting at 23.7% and 28% acetonitrile are  
17 named Peptide A and Peptide B, respectively. Similar  
18 results were obtained in four (A and B) and two (C)  
19 experiments.

20  
21 No major  $^{32}\text{P}$ -labelled peptides were recovered from  
22  $^{32}\text{P}$ -labelled PKB $\alpha$  derived from unstimulated L6 myotubes  
23 (Fig 6A) indicating that, in the absence of insulin,  
24 there was a low level phosphorylation at a number of  
25 sites. However, following stimulation with insulin, two  
26 major  $^{32}\text{P}$ -labelled peptides were observed, termed A and  
27 B (Fig 6B), whose  $^{32}\text{P}$ -labelling was prevented if the  
28 myotubes were first preincubated with wortmannin (Fig  
29 6C).

30  
31 Fig 7. Identification of the phosphorylation sites in  
32 peptides A and B. (A) Peptides A and B from Fig5B  
33 (1000cpm) were incubated for 90min at 110°C in 6M HCl,  
34 electrophoresed on thin layer cellulose at pH 3.5 to  
35 resolve orthophosphate (Pi), phosphoserine (pS),  
36 phosphthreonine (pT) and phosphotyrosine (pY) and

1 autoradiographed. (B) Peptide A (Fig 5B) obtained from  
2 50 10 cm dishes of  $^{32}\text{P}$ -labelled L6 myotubes was further  
3 purified by chromatography on a microbore C18 column  
4 equilibrated in 10 mM ammonium acetate pH 6.5 instead  
5 of 0.1% TFA. A single peak of  $^{32}\text{P}$ -radioactivity was  
6 observed at 21% acetonitrile which coincided with a  
7 peak of 214 nm absorbance. 80% of the sample (1 pmol)  
8 was analysed on an Applied Biosystems 476A sequencer to  
9 determine the amino acid sequence, and the  
10 phenylthiohydantoin (Pth) amino acids identified after  
11 each cycle of Edman degradation are shown using the  
12 single letter code for amino acids. The residues in  
13 parentheses were not present in sufficient amounts to  
14 be identified unambiguously. To identify the site(s)  
15 of phosphorylation, the remaining 20% of the sample  
16 (600 cpm) was then coupled covalently to a Sequelon  
17 arylamine membrane and analysed on an Applied  
18 Biosystems 470A sequencer using the modified programme  
19 described by Stokoe et al. (1992).  $^{32}\text{P}$  radioactivity was  
20 measured after each cycle of Edman degradation. (C)  
21 Peptide B from Fig 2B (800 cpm) was subjected to solid  
22 phase sequencing as in (B).

23

24 Peptide A was phosphorylated predominantly on serine  
25 while peptide B was labelled on threonine (Fig 7A).  
26 Amino acid sequencing established that peptide A  
27 commenced at residue 465. Only a single burst of  
28  $^{32}\text{P}$ -radioactivity was observed after the eighth cycle of  
29 Edman degradation (Fig 7B), demonstrating that insulin  
30 stimulation of L6 myotubes had triggered the  
31 phosphorylation of PKB $\alpha$  at Ser-473, which is located 9  
32 residues from the C-terminus of the protein.  
33 Phosphopeptide B was only recovered in significant  
34 amounts if  $^{32}\text{P}$ -labelled PKB $\alpha$  was treated with  
35 4-vinylpyridine prior to digestion with trypsin,  
36 indicating that this peptide contained a cysteine

1 residue(s), and a single burst of 32p radioactivity was  
2 observed after the first cycle of Edman degradation  
3 (Fig 7C). This suggested that the site of  
4 phosphorylation was residue 308, since it is the only  
5 threonine in PKB $\alpha$  that follows a lysine or arginine  
6 residue and which is located in a tryptic peptide  
7 containing a cysteine residue (at position 310). The  
8 acetonitrile concentration at which phosphopeptide B is  
9 eluted from the C18 column (28%) and its isoelectric  
10 point (4.0) are also consistent with its assignment as  
11 the peptide comprising residues 308-325 of PKB $\alpha$ . The  
12 poor recoveries of Peptide B during further  
13 purification at pH 6.5 prevented the determination of  
14 its amino acid sequence, but further experiments  
15 described below using transiently transfected 293 cells  
16 established that this peptide does correspond to  
17 residues 308-325 of PKB $\alpha$ .

18  
19 Fig 8: Mapping the phosphorylation sites of PKB $\alpha$  in  
20 transiently transfected 293 cells. 293 cells were  
21 transiently transfected with DNA constructs expressing  
22 wild type PKB $\alpha$ , or a haemagglutinin epitope-tagged PKB $\alpha$   
23 encoding the human protein, such as HA-KD PKB $\alpha$ , HA-473A  
24 PKB $\alpha$  or HA-308A PKB $\alpha$ . After treatment for 10 min with  
25 or without 100 nM wortmannin, the cells were stimulated  
26 for 10 min with or without 100 nM insulin or 50 ng/ml  
27 IGF- 1 in the continued presence of wortmannin. PKB $\alpha$   
28 was immunoprecipitated from the lysates and assayed,  
29 and activities corrected for the relative levels of  
30 expression of each HA-PKB $\alpha$ . The results are expressed  
31 relative to the specific activity of wild type HA-PKB $\alpha$   
32 from unstimulated 293 cells ( $2.5 \pm 0.5$  U/mg). (B) 20  $\mu$ g  
33 of protein from each lysate was electrophoresed on a 10  
34 % SDS/polyacrylamide gel and immunoblotted using  
35 monoclonal HA-antibody. The molecular markers are those  
36 used in Fig 5B.



1 Fig 9: IGF-1 stimulation of 293 cells induces the  
2 phosphorylation of two peptides in transfected HA-PKB $\alpha$ .  
3 293 cells transiently transfected with wild type HAPKB $\alpha$   
4 DNA constructs were  $^{32}$ P-labelled, treated for 10 min  
5 without (A,B) or with (C) 100 nM wortmannin and then  
6 for 10 min without (A) or with (B, C) 50 ng/ml IGF-1.  
7 The  $^{32}$ p labelled HA-PKB $\alpha$  was immunoprecipitated from  
8 the lysates, treated with 4-vinylpyridine,  
9 electrophoresed on a 10% polyacrylamide gel, excised  
10 from the gel and digested with trypsin. Subsequent  
11 chromatography on a C<sub>18</sub> column resolved four major  
12 phosphopeptides termed C, D, E and F. Similar results  
13 were obtained in 6 separate experiments for (A) and  
14 (B), and in two experiments for (C).

15  
16 Stimulation with insulin and IGF-1 resulted in 20-fold  
17 and 46-fold activation of transfected PKB $\alpha$ ,  
18 respectively (Fig 8A), the half time for activation  
19 being 1 min, as found with other cells. Activation of  
20 PKB $\alpha$  by insulin or IGF-1 was prevented by prior  
21 incubation with wortmannin (Fig 8A) and no activation  
22 occurred if 293 cells were transfected with vector  
23 alone and then stimulated with insulin or IGF-1 (data  
24 not shown).

25  
26 Two prominent  $^{32}$ P-labelled peptides were present in  
27 unstimulated 293 cells (Fig 9A). One, termed Peptide C,  
28 usually eluted as a doublet at 20-21% acetonitrile  
29 while the other, termed Peptide F, eluted at 29.7%  
30 acetonitrile. Stimulation with insulin or IGF-1 did  
31 not affect the  $^{32}$ P-labelling of Peptides C and F (Figs  
32 9A & B), but induced the  $^{32}$ P-labelling of two new  
33 peptides, termed D (23.4% acetonitrile) and E  
34 (28% acetonitrile), which eluted at the same  
35 acetonitrile concentrations as peptides A and B from L6  
36 myotubes (Fig 6B) and had the same isoelectric points

1 (7.2 and 4.0, respectively). Treatment of 293 cells  
2 expressing HA-PKB $\alpha$  with 100 nM wortmannin, prior to  
3 stimulation with IGF-1, prevented the phosphorylation  
4 of Peptides D and E, but had no effect on the <sup>32</sup>P  
5 labelling of Peptides C and F (Fig 9C).

6  
7 Peptides C, D, E and F were further purified by re-  
8 chromatography on the C18 column at pH 6.5 and  
9 sequenced. Peptides C gave rise to three separate (but  
10 closely eluting) <sup>32</sup>P-labelled peptides (data not shown).  
11 Amino acid sequencing revealed that all three commenced  
12 at residue 122 of PKB $\alpha$  and that Ser-124 was the site of  
13 phosphorylation (Fig 10A). Peptide D only contained  
14 phosphoserine and, as expected, corresponded to the  
15 PKB $\alpha$  tryptic peptide commencing at residue 465 that was  
16 phosphorylated at Ser-473 (Fig 10B). Peptide E, only  
17 contained phosphothreonine and amino acid sequencing  
18 demonstrated that it corresponded to residues 308-325,  
19 the phosphorylation site being Thr-308 (Fig 10C).  
20 Peptide F only contained phosphothreonine and  
21 corresponded to the peptide commencing at residue 437  
22 of PKB $\alpha$  phosphorylated at Thr-450 (Fig 10D).

23  
24 In the presence of phosphatidylserine, PKB $\alpha$  binds to  
25 PIP3 with submicromolar affinity (James et al., 1996,  
26 Frech et al., 1996). Phosphatidyl 4,5-bisphosphate and  
27 phosphatidyl 3,4 bisphosphate bind to PKB $\alpha$  with lower  
28 affinities and PI 3,5 bisphosphate and PI 3 phosphate  
29 did not bind at all under these conditions (James et  
30 al., 1996). The region of PKB $\alpha$  that interacts with PIP3  
31 is almost certainly the PH domain, because the isolated  
32 PH domain binds PIP3 with similar affinity to PKB $\alpha$   
33 itself (Frech et al., 1996) and because the PH domain  
34 of several other proteins, such as the PH-domains of,  
35  $\beta$ -spectrin and phospholipase C1, are known to interact  
36 specifically with other phosphoinositides (Hyvonen et

1 al., 1995 and Lemmon et al., 1995).

2  
3 The experiments described above were repeated using  
4 insulin instead of IGF-1. The results were identical,  
5 except that the  $^{32}\text{P}$ -labelling of Peptides D and E was  
6 about 50% of the levels observed with IGF-1 (data not  
7 shown). This is consistent with the two-fold lower  
8 level of activation of PKB $\alpha$  by insulin compared with  
9 IGF-1 (Fig 7A).

10

11 Example 3: MAPKAP kinase-2 phosphorylates Ser-473 of  
12 PKB $\alpha$  causing partial activation. Ser-473 of PKB $\alpha$  lies  
13 in a consensus sequence Phe-x-x-Phe/Tyr-Ser/Thr-Phe/Tyr  
14 found to be conserved in a number of protein kinases  
15 that participate in signal transduction pathways  
16 (Pearson et al. 1995). In order to identify the Ser-473  
17 kinase(s) we therefore chromatographed rabbit skeletal  
18 muscle extracts on CM-Sephadex, and assayed the  
19 fractions for protein kinases capable of  
20 phosphorylating a synthetic peptide corresponding to  
21 residues 465 to 478 of PKB $\alpha$ . These studies identified  
22 an enzyme eluting at 0.3 M NaCl which phosphorylated  
23 the peptide 465-478 at the residue equivalent to  
24 Ser-473 of PKB $\alpha$ . The Ser473 kinase co-eluted from  
25 CM-Sephadex with MAP kinase-activated protein (MAPKAP)  
26 kinase-2, (Stokoe et al, 1992) which is a component of  
27 a stress and cytokine-activated MAP kinase cascade  
28 (Rouse et al, 1994; Cuenda et al, 1995). The Ser-473  
29 kinase continued to cofractionate with MAPKAPkinase-2  
30 through phenyl-Sepharose, heparin-Sepharose, Mono S and  
31 Mono Q and was immunoprecipitated quantitatively by an  
32 anti-MAPKAP kinase-2 antibody (Gould et al, 1995)  
33 demonstrating that MAPKAP kinase-2 was indeed the  
34 Ser-473 kinase we had purified.

35

36 Figure 11. HA-PKB $\alpha$  was immunoprecipitated from the

1 lysates of unstimulated COS-1 cells expressing these  
2 constructs. (A) 0.5  $\mu$ g of immunoprecipitated HA-PKB $\alpha$   
3 was incubated with MAPKAP kinase-2 (50 U/ml), 10 mM  
4 magnesium acetate and 100 mM [ $\gamma$ <sup>32</sup>P]ATP in a total of 40  
5  $\mu$ l of Buffer B. At various times, aliquots were removed  
6 and either assayed for PKB $\alpha$  activity (open circles) or  
7 for incorporation of phosphate into PKB $\alpha$  (closed  
8 circles). Before measuring PKB $\alpha$  activity, EDTA was  
9 added to a final concentration of 20 mM to stop the  
10 reaction, and the immunoprecipitates washed twice with  
11 1.0 ml of buffer B containing 0.5 M NaCl, then twice  
12 with 1.0 ml of Buffer B to remove MAPKAP kinase-2. The  
13 results are presented as  $\pm$  SEM for six determinations  
14 (two separate experiments) and PKB $\alpha$  activities are  
15 presented relative to control experiments in which  
16 HA-PKB $\alpha$  was incubated with MgATP in the absence of  
17 MAPKAP kinase-2 (which caused no activation).  
18 Phosphorylation was assessed by counting the  
19 <sup>32</sup>P-radioactivity associated with the band of PKB $\alpha$  after  
20 SDS/polyacrylamide gel electrophoresis. The open  
21 triangles show the activity of immunoprecipitated HA-KD  
22 PKB $\alpha$  phosphorylated by MAPKAP kinase-2. (B) HA-PKB $\alpha$   
23 phosphorylated for 1 h with MAPKAP kinase-2 and <sup>32</sup>P-  
24  $\gamma$ -ATP as in (A) was digested with trypsin and  
25 chromatographed on a C18 column as described in the  
26 legend for Fig 2. (C) The major <sup>32</sup>P-labelled peptide  
27 from (B) was analysed on the 470A sequencer as in Fig 3  
28 to identify the site of phosphorylation.

29

30 Bacterially expressed MAPKAP kinase-2 phosphorylated  
31 wild type HA-PKB $\alpha$  or the catalytically inactive mutant  
32 HA-PKB $\alpha$  in which Lys- 179 had been mutated to Ala (data  
33 not shown) to a level approaching 1 mol per mole  
34 protein (Fig 11A). Phosphorylation of wild-type PKB $\alpha$   
35 was paralleled by a seven-fold increase in activity,  
36 whereas phosphorylation of the catalytically inactive

1 mutant did not cause any activation (Fig 11A). No  
2 phosphorylation or activation of wild type HA-PKB $\alpha$   
3 occurred if MAPKAP kinase-2 or MgATP was omitted from  
4 the reaction (data not shown). Wild type HA-PKB $\alpha$  that  
5 had been maximally activated with MAPKAP kinase-2, was  
6 completely dephosphorylated and inactivated by  
7 treatment with protein phosphatase 2A (data not shown).

8  
9 HA-PKB $\alpha$  that had been maximally phosphorylated with  
10 MAPKAP kinase-2 was digested with trypsin and C18  
11 chromatography revealed a single major  $^{32}\text{P}$ -labelled  
12 phosphoserine-containing peptide (Fig 11B). This  
13 peptide eluted at the same acetonitrile concentration  
14 (Fig 11B) and had the same isoelectric point of 7.2  
15 (data not shown) as the  $^{32}\text{P}$  labelled tryptic peptide  
16 containing Ser-473 (compare Figs 11B and 6B). Solid  
17 phase sequencing gave a burst of  $^{32}\text{P}$ -radioactivity after  
18 the eighth cycle of Edman degradation (Fig 11C),  
19 establishing that Ser-473 was the site of  
20 phosphorylation. The same  $^{32}\text{P}$ -peptide was obtained  
21 following tryptic digestion of catalytically inactive  
22 HA-KD PKB $\alpha$  that had been phosphorylated with MAPKAP  
23 kinase-2 (data not shown).

24  
25 Example 4: Phosphorylation of Thr-308 and Ser-473  
26 causes synergistic activation of PKB $\alpha$ . The experiments  
27 described above demonstrated that phosphorylation of  
28 Ser-473 activates PKB $\alpha$  in vitro but did not address the  
29 role of phosphorylation of Thr-308, or how  
30 phosphorylation of Thr-308 might influence the effect  
31 of Ser-473 phosphorylation on activity, or vice versa.  
32 We therefore prepared haemagglutinin (HA)-tagged PKB $\alpha$   
33 DNA constructs in which either Ser-473 or Thr-308 would  
34 be changed either to Ala (to block the effect of  
35 phosphorylation) or to Asp (to try and mimic the effect  
36 of phosphorylation).

1 Fig 12. Activation of HA-PKB $\alpha$  mutants in vitro by  
2 MAPKAP kinase-2. (A) Wild type and mutant HA-PKB $\alpha$   
3 proteins were immunoprecipitated from the lysates of  
4 unstimulated COS-1 cells expressing these constructs  
5 and incubated for 60 min with MgATP in the absence (-,  
6 filled bars) or presence (+, hatched bars) of MAPKAP  
7 kinase-2 and MgATP (50 U/ml). The PKB $\alpha$  protein was  
8 expressed as similar levels in each construct and  
9 specific activities are presented relative to wild type  
10 HA-PKB $\alpha$  incubated in the absence of MAPKAP kinase-2  
11 (0.03 U/mg). The results are shown as the average  $\pm$  SEM  
12 for 3 experiments. (B ) 20  $\mu$ g of protein from each  
13 lysate was electrophoresed on a 10 % SDS/polyacrylamide  
14 gel and immunoblotted using monoclonal HA-antibody.

15  
16 All the mutants were expressed at a similar level in  
17 serum-starved COS-1 cells (data not shown) and the  
18 effects of maximally phosphorylating each of them at  
19 Ser-473 is shown in Fig 12A. Before phosphorylation  
20 with MAPKAP kinase-2 the activity of HA-473A PKB $\alpha$  was  
21 similar to that of unstimulated wild type HA-PKB $\alpha$  and,  
22 as expected, incubation with MAPKAP kinase-2 and MgATP  
23 did not result in any further activation of HA-473A  
24 PKB $\alpha$ . In contrast, the activity of HA-473D PKB $\alpha$  was  
25 five-fold to six-fold higher than that of unstimulated  
26 wild type HAPKB $\alpha$  protein, and similar to that of  
27 wild-type HA-PKB $\alpha$  phosphorylated at Ser-473. As  
28 expected, HA-473D PKB $\alpha$  was also not activated further  
29 by incubation with MAPKAP kinase-2 and MgATP. The  
30 activity of HA-308A PKB $\alpha$  was about 40% that of the  
31 unstimulated wild type enzyme, and after  
32 phosphorylation with MAPKAP kinase-2 is activity  
33 increased to a level similar to that of wild type  
34 HA-PKB $\alpha$  phosphorylated at Ser-473. Interestingly,  
35 HA-308D PKB $\alpha$  which (like HA-473D PK) was five-fold more  
36 active than dephosphorylated wild type HA-PKB $\alpha$ , was

1 activated dramatically by phosphorylation of Ser-473.  
2 After incubation with MAPKAP kinase-2 and MgATP, the  
3 activity of HA-308D PKB $\alpha$  was nearly five-fold higher  
4 than that of wild type HA-PKB $\alpha$  phosphorylated at  
5 Ser-473 (Fig 12B). These results suggested that the  
6 phosphorylation of either Thr-308 or Ser-473 leads to  
7 partial activation of PKB $\alpha$  in vitro, and that  
8 phosphorylation of both residues results in a  
9 synergistic activation of the enzyme. This idea was  
10 supported by further experiments in which both Thr-308  
11 and Ser-473 were changed to Asp. When this double  
12 mutant was expressed in COS-1 cells it was found to  
13 possess an 18-fold higher specific activity than the  
14 dephosphorylated wild type protein. As expected, the  
15 activity of this mutant was not increased further by  
16 incubation with MAPKAP kinase-2 and MgATP (Fig 12B).

17  
18 Example 5: Phosphorylation of both Thr-308 and Ser-473  
19 is required for a high level of activation of PKB $\alpha$  in  
20 vivo.

21  
22 Fig 9. Effect of mutation of PKB $\alpha$  on its activation by  
23 insulin in 293 cells. (A) 293 cells were transiently  
24 transfected with DNA constructs expressing wild type  
25 PKB $\alpha$ , HA-D473- PKB $\alpha$ , and HA-308D/473D-PKB $\alpha$ . After  
26 treatment for 10 min with or without 100 nM wortmannin,  
27 cells were stimulated for 10 min with or without 100 nM  
28 insulin in the continued presence of wortmannin. PKB $\alpha$   
29 was immunoprecipitated from the lysates and assayed,  
30 and activities corrected for the relative levels of  
31 HA-PKB $\alpha$  expression as described in the methods. The  
32 results are expressed relative to the specific activity  
33 of wild type HA-PKB $\alpha$  obtained from unstimulated 293  
34 cells. (B) 20  $\mu$ g of protein from each lysate was  
35 electrophoresed on a 10 % SDS/polyacrylamide gel and  
36 immunoblotted using monoclonal HA-antibody.

1 The basal level of activity of HA-473A PKB $\alpha$  derived  
2 from unstimulated cells was similar to that of wild  
3 type PKB $\alpha$  (Fig 8A). Stimulation of 293 cells expressing  
4 HA-473A PKB $\alpha$  with insulin or IGF-1 increased the  
5 activity of this mutant three-fold and five-fold  
6 respectively; i.e. to 15% of the activity of wild type  
7 HA-PKB $\alpha$  which had been transiently expressed and  
8 stimulated under identical conditions. The basal  
9 activity of HA-308A PKB $\alpha$  in unstimulated cells was also  
10 similar to that of wild type HA-PKB $\alpha$  derived from  
11 unstimulated cells, but virtually no activation of this  
12 mutant occurred following stimulation of the cells with  
13 insulin or IGF-1. These data are consistent with in  
14 vitro experiments and indicate that maximal activation  
15 of PKB $\alpha$  requires phosphorylation of both Ser-473 and  
16 Thr-308 and that phosphorylation of both residues  
17 results in a synergistic activation of the enzyme.  
18 Consistent with these results, HA-473D PKB $\alpha$  displayed  
19 five-fold higher activity and the HA-308D/HA473D double  
20 mutant 40-fold higher activity than wild type HA-PKB $\alpha$   
21 when expressed in unstimulated cells. Following  
22 stimulation with insulin, HA-473D PKB $\alpha$  was activated to  
23 a level similar to that observed with the wildtype  
24 enzyme, while the HA-308D/HA-473D double mutant could  
25 not be activated further (Fig 13). As expected,  
26 activation of HA-473D PKB $\alpha$  by insulin was prevented by  
27 wortmannin, and the activity of the HA-308D/ HA-473D  
28 double mutant was resistant to wortmannin (Fig 13).

29  
30 Example 6: Phosphorylation of Thr-308 is not dependent  
31 on phosphorylation of Ser-473 or vice versa (in 293  
32 cells). (Fig 10) A 10 cm dish of 293 cells were  
33 transfected with either HA-308A PKB $\alpha$  or HA-473A PKB $\alpha$ ,  
34 <sup>32</sup>P-labelled, then stimulated for 10 min with either  
35 IGF-1 (50 ng/ml) or buffer. The <sup>32</sup>P-labelled PKB $\alpha$   
36 mutants were immunoprecipitated from the lysates,



1 treated with 4-vinylpyridine, electrophoresed on a 10%  
2 polyacrylamide gel, excised from the gel and digested  
3 with trypsin, then chromatographed on a C18 column.  
4 The tryptic peptides containing the phosphorylated  
5 residues Ser-124, Thr-308, Thr-450, Ser-473 are marked  
6 and their assignments were confirmed by phosphoamino  
7 acid analysis and sequencing to identify the sites of  
8 phosphorylation (data not shown). The phosphopeptides  
9 containing Thr-308 and Ser-473 were absent if  
10 stimulation with IGF-1 was omitted, while the  
11 phosphopeptides containing Ser-124 and Thr-450 were  
12 present at similar levels as observed with wild-type  
13 PKB $\alpha$  (see Fig 9A). Similar results were obtained in 3  
14 separate experiments.

15  
16 These experiments demonstrated that IGF-1 stimulation  
17 induced the phosphorylation of HA-473A PKB $\alpha$  at Thr-308,  
18 and the phosphorylation of HA-308A PKB $\alpha$  at Ser-473.  
19 Similar results were obtained after stimulation with  
20 insulin rather than IGF-I.

21  
22 Example 7: IGF-1 or insulin induces phosphorylation of  
23 Thr-308 and Ser-473 in a catalytically inactive mutant  
24 of PKB $\alpha$ .

25  
26 Fig 15. The catalytically inactive PKB $\alpha$  mutant  
27 (HA-KD-PKB $\alpha$ ) expressed in 293 cells is phosphorylated  
28 at Thr-308 and Ser-473 after stimulation with IGF-1.  
29 Each 10 cm dish of 293 cells transiently transfected  
30 with HA-KD-PKB $\alpha$  DNA constructs was <sup>32</sup>P-labelled and  
31 incubated for 10 min with buffer (A), 50 ng/ml IGF-1  
32 (B) or 100 nM insulin (C). The <sup>32</sup>P-labelled HA-KD-PKB $\alpha$   
33 was immunoprecipitated from the lysates, treated with 4  
34 vinylpyridine, electrophoresed on a 10% polyacrylamide  
35 gel, excised from the gel and digested with trypsin,  
36 then chromatographed on a C18 column. The tryptic

1 peptides containing the phosphorylated residues  
2 Ser-124, Thr-308, Thr-450 and Ser-473 are marked.  
3 Similar results were obtained in 3 separate experiments  
4 for (A) and (B), and in two experiments for (C).  
5

6 This kinase dead" mutant of PKB $\alpha$ , termed HA-KD-PKB $\alpha$ , in  
7 which Lys-179 was changed to Ala (see above) was  
8 transiently expressed in 293 cells and its level of  
9 expression found to be several-fold lower than that of  
10 wild type HA-PKB $\alpha$  expressed under identical conditions  
11 (Fig 8B). As expected, no PKB $\alpha$  activity was detected  
12 when 293 cells expressing HA-KD-PKB $\alpha$ , were stimulated  
13 with insulin or IGF-1 (Fig 7A).  
14

15 293 cells that had been transiently transfected with  
16 HA-KD-PKB $\alpha$  were  $^{32}$ P-labelled, then stimulated with  
17 buffer, insulin or IGF-1. and sites on PKB $\alpha$   
18 phosphorylated under these conditions were mapped. In  
19 contrast to wild type HA-PKB $\alpha$  from unstimulated 293  
20 cells (Fig 9), HA-KD PKB $\alpha$  was phosphorylated to a much  
21 lower level at Ser-124, but phosphorylated similarly at  
22 Thr-450 (Fig 15A). Following stimulation with IGF-1  
23 (Fig 15B) or insulin (Fig 14C) HA-KD-PKB $\alpha$  became  
24 phosphorylated at the peptides containing Thr-308 and  
25 Ser-473, the extent of phosphorylation of these sites  
26 being at least as high as wild type PKB $\alpha$ . Amino acid  
27 sequencing of these peptides established that they were  
28 phosphorylated at Thr-308 and Ser-473, respectively.  
29

30 The above examples establish that PKB influences GSK3  
31 activity; that Thr-308 and Ser-473 are the major  
32 residues in PKB $\alpha$  that become phosphorylated in response  
33 to insulin or IGF-1 (Figs 2 and 5) and that  
34 phosphorylation of both residues is required to  
35 generate a high level of PKB $\alpha$  activity. Thus mutation  
36 of either Thr-308 or Ser-473 to Ala greatly decreased

1 the activation of transfected PKB $\alpha$  by insulin or IGF-1  
2 in 293 cells (Fig 8). Moreover, PKB $\alpha$  became partially  
3 active in vitro when either Thr-308 or Ser-473 were  
4 changed to Asp or when Ser-473 was phosphorylated by  
5 MAPKAP kinase-2 in vitro, and far more active when the  
6 D308 mutant of PKB $\alpha$  was phosphorylated by MAPKAP  
7 kinase-2 or when Thr-308 and Ser-473 were both mutated  
8 to Asp (Fig 12). Moreover, the D308/D473 double mutant  
9 could not be activated further by stimulating cells  
10 with insulin (Fig 13). These observations demonstrate  
11 that the phosphorylation of Thr-308 and Ser-473 act  
12 synergistically to generate a high level of PKB $\alpha$   
13 activity.

14  
15 Thr-308, and the amino acid sequence surrounding it, is  
16 conserved in rat PKB $\beta$  and PKB $\gamma$  but, interestingly,  
17 Ser-473 (and the sequence surrounding it) is only  
18 conserved in PKB $\beta$ . In rat PKB $\gamma$ , Ser-473 is missing  
19 because the C-terminal 23 residues are deleted. This  
20 suggests that the regulation of PKB $\gamma$  may differ  
21 significantly from that of PKB $\alpha$  and PKB $\beta$  in the rat.

22  
23 Thr-308 is located in subdomain VIII of the kinase  
24 catalytic domain, nine residues upstream of the  
25 conserved Ala-Pro-Glu motif, the same position as  
26 activating phosphorylation sites found in many other  
27 protein kinases. However, Ser-473 is located C-terminal  
28 to the catalytic domain in the consensus sequence  
29 Phe-Xaa-Xaa-Phe/Tyr-Ser/Thr-Phe/Tyr which is present in  
30 several protein kinases that participate in growth  
31 factor-stimulated kinase cascades, such as p70 S6  
32 kinase, PKC and p90rsk (Pearson et al, 1995). However,  
33 it is unlikely that a common protein kinase  
34 phosphorylates this motif in every enzyme for the  
35 following reasons. Firstly, phosphorylation of the  
36 equivalent site in p70 S6 kinase is prevented by the

1 immunosuppressant drug rapamycin (Pearson et al, 1995)  
2 which does not prevent the activation of PKB $\alpha$  by  
3 insulin (Cross et al, 1995) or is phosphorylation at  
4 Ser-473 (D. Alessi, unpublished work). Secondly, the  
5 equivalent residue in protein kinase C is  
6 phosphorylated constitutively and not triggered by  
7 stimulation with growth factors (Tsutakawa et al.,  
8 1995).  
9  
10 MAPKAP kinase-2 is a component of a protein kinase  
11 cascade which becomes activated when cells are  
12 stimulated with interleukin-1 or tumour necrosis factor  
13 or exposed cellular stresses (Rouse et al, 1994; Cuenda  
14 et al, 1995). MAPKAP kinase-2 phosphorylates PKB $\alpha$   
15 stoichiometrically at Ser-473 (Fig 11) and this finding  
16 was useful in establishing the role of Ser473  
17 phosphorylation in regulating PKB $\alpha$  activity. However,  
18 although MAPKAP kinase-2 activity is stimulated to a  
19 small extent by insulin in L6 cells, no activation  
20 could be detected in 293 cells in response to insulin  
21 or IGF-1. Moreover, exposure of L6 cells or 293 cells  
22 to a chemical stress (0.5 mM sodium arsenite) strongly  
23 activated MAPKAP kinase-2 (D. Alessi, unpublished work)  
24 as found in other cells (Rouse et al, 1994; Cuenda et  
25 al, 1995), but did not activate PKB $\alpha$  at all.  
26 Furthermore, the drug SB 203580 which is a specific  
27 inhibitor of the protein kinase positioned immediately  
28 upstream of MAPKAP kinase-2 (Cuenda et al, 1995),  
29 prevented the activation of MAPKAP kinase-2 by arsenite  
30 but had no effect on the activation of PKB $\alpha$  by insulin  
31 or IGF-1. Finally, the activation of PKB $\alpha$  was prevented  
32 by wortmannin (Figs 6 and 9), but wortmannin had no  
33 effect on the activation of MAPKAP kinase-2 in L6 or  
34 293 cells. It should also be noted that the sequence  
35 surrounding Ser-473 of PKB $\alpha$  (HFPQFSY) does not conform  
36 to the optimal consensus for phosphorylation by MAPKAP

1 kinase-2 which requires Arg at position n-3 and a bulky  
2 hydrophobic residue at position n-5, (where n is the  
3 position of the phosphorylated residue). The  $K_m$  for  
4 phosphorylation of the synthetic peptide comprising  
5 residues 465-478 is nearly 100-fold higher than the  $K_m$   
6 for the standard MAPKAP kinase-2 substrate peptide  
7 (data not shown). It is therefore unlikely that MAPKAP  
8 kinase-2 mediates the phosphorylation of Ser-473 in  
9 vivo.

10

11 The enzyme(s) which phosphorylates Thr-308 and Ser-473  
12 in vivo does not appear to be PKB $\alpha$  itself. Thus  
13 incubation of the partially active Asp-308 mutant with  
14 MgATP did not result in the phosphorylation of Ser-473,  
15 phosphorylation of the latter residue only occurring  
16 when MAPKAP kinase-2 was added (Fig 11A, Fig 12).  
17 Similarly, Thr-308 did not become phosphorylated when  
18 either the partially active D473 mutant or the  
19 partially active Ser-473 phosphorylated form of PKB $\alpha$   
20 were incubated with MgATP. PKB $\alpha$  when bound to lipid  
21 vesicles containing phosphatidylserine and PIP3 also  
22 fails to activate upon incubation with MgATP (Alessi et  
23 al, 1996) and after transfection into 293 cells, a  
24 "kinase dead" mutant of PKB $\alpha$  became phosphorylated on  
25 Thr-308 and Ser-473 in response to insulin or IGF-1  
26 (Fig 14). Furthermore, HA-PKB $\alpha$  from either unstimulated  
27 or insulin-stimulated 293 cells failed to phosphorylate  
28 the synthetic C-terminal peptide comprising amino acids  
29 467-480.

30

31 In unstimulated L6 myotubes, the endogenous PKB $\alpha$  was  
32 phosphorylated at a low level at a number of sites (Fig  
33 6A), but in unstimulated 293 cells the transfected  
34 enzyme was heavily phosphorylated at Ser-124 and  
35 Thr-450 (Fig 10). Ser-124 and Thr-450 are both followed  
36 by proline residues suggesting the involvement of

1 "proline-directed" protein kinases. Although, the  
2 phosphorylation of Ser-124 was greatly decreased when  
3 "kinase dead" PKB $\alpha$  was transfected into 293 cells (Fig  
4 14), it would be surprising if Ser-124 is  
5 phosphorylated by PKB $\alpha$  itself because the presence of a  
6 C-terminal proline abolishes the phosphorylation of  
7 synthetic peptides by PKB $\alpha$  (D.Alessi, unpublished  
8 work). Since transfected PKB $\alpha$  is inactive in  
9 unstimulated 293 cells (Fig 12), phosphorylation of  
10 Ser-124 and Thr-450 clearly does not activate PKB $\alpha$   
11 directly. Ser-124 is located in the linker region  
12 between the PH domain and the catalytic domain of the  
13 mammalian PKB $\alpha$  isoforms but, unlike Thr-450, is not  
14 conserved in the Drosophila homologue (Andjelkovic et  
15 al, 1995).

16  
17 While we do not wish to be bound by hypotheses, the  
18 results described suggest that agonists which activate  
19 PI 3-kinase are likely to stimulate PKB $\alpha$  activity via  
20 one of the following mechanisms. Firstly, PIP3 or  
21 PI3,4-bisP may activate one or more protein kinases  
22 which then phosphorylate PKB $\alpha$  at Thr-308 and Ser-473.  
23 Secondly, the formation of PIP3 may lead to the  
24 recruitment of PKB $\alpha$  to the plasma membrane where it is  
25 activated by a membrane-associated protein kinase(s).  
26 The membrane associated Thr-308 and Ser-473 kinases  
27 might themselves be activated by PIP3 and the  
28 possibility that Thr-308 and/or Ser-473 are  
29 phosphorylated directly by PI 3-kinase has also not  
30 been excluded, because this enzyme is known to  
31 phosphorylate itself (Dhand et al, 1994) and other  
32 proteins (Lam et al, 1994) on serine residues.

33

34 Example 8: Molecular basis for substrate specificity of  
35 PKB. PKB $\alpha$  has been shown to influence GSK3 activity.  
36 GSK3 $\alpha$  and GSK3 $\beta$  are phosphorylated at Ser-21 and Ser-9,

1     respectively, by two other insulin-stimulated protein  
2     kinases, namely p70 S6 kinase and MAP kinase-activated  
3     protein kinase-1 (MAPKAP-K1, also known as p90 S6  
4     kinase). However, these enzymes are not rate-limiting  
5     for the inhibition of GSK3 by insulin in L6 myotubes  
6     because specific inhibitors of their activation  
7     (rapamycin-p70 S6 kinase; PD 98059-MAPKAP kinase-1)  
8     have no effect (Cross et al., 1995).

9  
10    The activation of PI 3-kinase is essential for many of  
11    the effects of insulin and growth factors, including  
12    the stimulation of glucose transport, fatty acid  
13    synthesis and DNA synthesis, protection of cells  
14    against apoptosis and actin cytoskeletal rearrangements  
15    (reviewed in Carpenter et al., 1996). These  
16    observations raise the question of whether PKB $\alpha$   
17    mediates any of these events by phosphorylating other  
18    proteins. To address this issue we characterised the  
19    substrate specificity requirements of PKB $\alpha$ . We find  
20    that the optimal consensus sequence for phosphorylation  
21    by PKB $\alpha$  is the motif Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd,  
22    where Yaa and Zaa are small amino acids (other than  
23    glycine) and Hyd is a large hydrophobic residue (such  
24    as Phe or Leu). We also demonstrate that PKB $\alpha$   
25    phosphorylates histone H2B (a substrate frequently used  
26    to assay PKB $\alpha$  in vitro) at Ser-36 which lies in an Arg-  
27    Xaa-Arg-Xaa-Xaa-Ser-Hyd motif. These studies identified  
28    a further PKB $\alpha$  substrate (Arg-Pro-Arg-Ala-Ala-Thr-Phe)  
29    that, unlike other peptides, is not phosphorylated to a  
30    significant extent by either p70 S6 kinase or MAPKAP-  
31    K1.

## 32 33 34    Results

### 35    Preparation of Protein Kinase B $\alpha$

36    In order to examine the substrate specificity of PKB $\alpha$ ,

1 it was first necessary to obtain a kinase preparation  
2 that was not contaminated with any other protein kinase  
3 activities. 293 cells were therefore transiently  
4 transfected with a DNA construct expressing  
5 haemagglutinin-tagged PKB $\alpha$  (HA-PKB $\alpha$ ), stimulated with  
6 IGF-1 and the HA-PKB $\alpha$  immunoprecipitated from the  
7 lysates). IGF-1 stimulation resulted in a 38-fold  
8 activation of PKB $\alpha$  (Fig 16) and analysis of the  
9 immunoprecipitates by SDS-polyacrylamide gel  
10 electrophoresis revealed that the 60 kDa PKB $\alpha$  was the  
11 major protein staining with Coomassie Blue apart from  
12 the heavy and light chains of the haemagglutinin  
13 monoclonal antibody (Fig 16, Lanes 2 and 3). The minor  
14 contaminants were present in control immunoprecipitates  
15 derived from 293 cells transfected with an empty pCMV5  
16 vector but lacked HA-PKB activity (Fig 16, Lane 4).  
17 Furthermore, a catalytically inactive mutant HA-  
18 PKB $\alpha$  immunoprecipitated from the lysates of IGF-1  
19 stimulated 293 cells had no Crosstide kinase activity  
20 (Alessi et al., 1996). Thus, all the Crosstide activity  
21 in HA-PKB immunoprecipitates is catalysed by PKB $\alpha$ .

22

23 Identification of the residues in histone H2B  
24 phosphorylated by PKB $\alpha$ . Currently, three substrates are  
25 used to assay PKB $\alpha$  activity in different laboratories,  
26 histone H2B, MBP and Crosstide. PKB $\alpha$  phosphorylated  
27 Crosstide with a Km of 4  $\mu$ M and a Vmax of 260 U/mg  
28 (Table 7.1 A, peptide 1), histone H2B with a Km of 5  $\mu$ M  
29 and a Vmax of 68 U/mg, and MBP with a Km of 5  $\mu$ M and a  
30 Vmax of 25 U/mg. Thus the Vmax of histone H2B and MBP  
31 are 4-fold and 10-fold lower than for Crosstide. In  
32 order to identify the residue(s) in histone H2B  
33 phosphorylated by PKB $\alpha$ ,  $^{32}$ P-labelled histone H2B was  
34 digested with trypsin (see Methods) and the resulting  
35 peptides chromatographed on a C18 column at pH 1.9.  
36 Only one major  $^{32}$ P-labelled peptide (termed T1) eluting



1 at 19.5 % acetonitrile was observed (Fig 17A), The  
2 peptide contained phosphoserine (data not shown), its  
3 sequence commenced at residue 34 of histone H2B and a  
4 single burst of radioactivity occurred after the third  
5 cycle of Edman degradation (Fig 17B), demonstrating  
6 that PKB $\alpha$  phosphorylates histone H2B at Ser-36 within  
7 the sequence Arg-Ser-Arg-Lys-Glu-Ser-Tyr. Thus, like  
8 the serine phosphorylated in Crosstide, Ser-36 of  
9 histone H2B lies in an Arg-Xaa-Arg-Xaa-Xaa-Ser-Hyd  
10 motif (where Hyd is a bulky hydrophobic residue -Phe in  
11 Crosstide, Tyr in H2B).

12

### 13 Molecular basis for the substrate specificity of PKB $\alpha$

14 To further characterise the substrate specificity  
15 requirements for PKB $\alpha$ , we first determined the minimum  
16 sequence phosphorylated efficiently by PKB $\alpha$  by removing  
17 residues sequently from the C-terminal and N-terminal  
18 end of Crosstide. Removal of the N-terminal glycine and  
19 up to three residues from the C-terminus had little  
20 effect on the kinetics of phosphorylation by PKB $\alpha$   
21 (Table 7.1A, compare peptides 1 and 5). However any  
22 further truncation of either the N or C-terminus  
23 virtually abolished phosphorylation (Table 7.1A,  
24 peptides 8 and 9). The minimum peptide phosphorylated  
25 efficiently by PKB $\alpha$  (Arg-Pro-Arg-Thr-Ser-Ser-Phe) was  
26 found to be phosphorylated exclusively at the second  
27 serine residue as expected. Consistent with this  
28 finding, a peptide in which this serine was changed to  
29 alanine was not phosphorylated by PKB $\alpha$  (Table 7.1A,  
30 peptide 7). All further studies were therefore carried  
31 out using variants of peptide 5 in Table 7.1A (see  
32 below).

33

34 A peptide in which the second serine of peptide 5  
35 (Table 7.1A) was replaced by threonine was  
36 phosphorylated with a Km of 30  $\mu$ M and an unchanged Vmax

1 (Table 7.1, peptide 6). All the  $^{32}\text{P}$ -radioactivity  
2 incorporated was present as phosphothreonine and solid  
3 phase sequencing revealed that the peptide was only  
4 phosphorylated at the second threonine residue, as  
5 expected (data not shown). Thus PKB $\alpha$  is capable of  
6 phosphorylating threonine as well as serine residues,  
7 but has a preference for serine.

8  
9 We next changed either of the two arginine residues in  
10 peptide 5 to lysine. These substitutions drastically  
11 decreased the rate of phosphorylation by PKB $\alpha$  (Table  
12 7.1A, peptides 10 and 11) demonstrating a requirement  
13 for arginine (and not simply any basic residue) at both  
14 positions.

15  
16 We then examined the effect of substituting the  
17 residues situated immediately C-terminal to the  
18 phosphorylated serine in peptide 5 (Table 7.1B). The  
19 data clearly demonstrate that the presence of a large  
20 hydrophobic residue at this position is critical for  
21 efficient phosphorylation, with the  $K_m$  increasing  
22 progressively with decreasing hydrophobicity of the  
23 residue at this position (Table 7.1B, peptides 1 to 4).  
24 Replacement of the C-terminal residue with Lys  
25 increased the  $K_m$  18-fold and a substitution at this  
26 position with either Glu or Pro almost abolished  
27 phosphorylation (Table 7.1B, peptides 5-7).

28  
29 Replacement of the Thr situated two residues N-terminal  
30 to the phosphorylated serine increased the  $K_m$  with any  
31 amino acid tested (Table 7.1C). Substitution with Ala  
32 only increased  $K_m$  by 2-3 fold, but substitution with  
33 other residues was more deleterious and with Asn (a  
34 residue of similar size and hydrophilicity to Thr)  
35 phosphorylation was almost abolished (Table 7.1C).  
36 Replacement of the Ser situated one residue N-terminal

1 to the phosphorylated serine also increased the  $K_m$  with  
2 any amino acid tested, but the effects were less severe  
3 than at position n-2 (Table 7.1C). When residues n-2  
4 and n-1 were both changed to Ala, the resulting peptide  
5 RPRAASF was phosphorylated by PKB $\alpha$  with a  $K_m$  only 5-  
6 fold higher than RPRTSSF. In contrast the peptides  
7 RPRGGSF, RPRAGSF, and RPRGASF were phosphorylated less  
8 efficiently (Table 7.1C).

9  
10 Comparison of the substrate specificity of PKB $\alpha$  with  
11 MAPKAP kinase-1, and p70 S6 kinase. Since MAPKAP-K1  
12 and p70 S6 kinase phosphorylate the same residue in  
13 GSK3 phosphorylated by PKB $\alpha$ , and studies with synthetic  
14 peptides have established that MAPKAP-K1 and p70 S6  
15 kinase also preferentially phosphorylate peptides in  
16 which basic residues are present at positions n-3 and  
17 n-5 (Leighton et al., 1995), we compared the  
18 specificities of MAPKAP-K1, p70 S6 kinase and PKB $\alpha$  in  
19 greater detail.

20  
21 MAPKAP kinase-1 and p70 S6 kinase phosphorylate the  
22 peptides KKKNRTLSVA and KKRNRTLSVA with extremely low  
23  $K_m$  values of 0.2- 3.3  $\mu M$ , respectively (Table 7.2).  
24 However, these peptides were phosphorylated by PKB $\alpha$   
25 with 50-900 fold higher  $K_m$  values (Table 7.2A, peptides  
26 1 and 2). The peptide KKRNRTLTV, which is a relatively  
27 specific substrate for p70 S6 kinase (Leighton et al.,  
28 1995) was also phosphorylated very poorly by PKB $\alpha$   
29 (Table 7.2A, peptide 4).

30  
31 Crosstide is phosphorylated by p70 S6 kinase and MAPKAP  
32 kinase-1 with similar efficiency to PKB $\alpha$  ((Leighton et  
33 al., 1995); Table 7.2B-peptide 1 and Fig 18). However,  
34 truncation of Crosstide to generate the peptide RPRTSSF  
35 was deleterious for phosphorylation by MAPKAP-K1 and  
36 even worse for p70 S6 kinase (Table 7.2B-peptides 1 and

1 2 and Fig 18). Moreover, changing the phosphorylated  
2 serine in RPRTSSF to threonine increased the  $K_m$  for  
3 phosphorylation by p70 S6 kinase much more than for  
4 PKB $\alpha$  and almost abolished phosphorylation by MAPKAP-K1  
5 (Table 7.2B-peptide 3 and Fig 18). The peptide RPRAASF  
6 was phosphorylated by MAPKAP-K1 with essentially  
7 identical kinetics to that of PKB $\alpha$ ; however  
8 phosphorylation by p70 S6 kinase was virtually  
9 abolished (Table 7.2B-peptide 4 and Fig 18). Based on  
10 these observations we synthesized the peptide RPRAATF.  
11 This peptide was phosphorylated by PKB $\alpha$  with a  $K_m$  of  
12 25  $\mu$ M and similar  $V_{max}$  to RPRTSSF, but was not  
13 phosphorylated to a significant extent by either  
14 MAPKAP-K1 or p70 S6 kinase (Table 7.2B-peptide 5, Fig  
15 18). In Fig 18, the protein kinase concentration in  
16 the assays towards Crosstide were 0.2 U/ml, and each  
17 peptide substrate was assayed at a concentration of 30  
18  $\mu$ M. Filled bars denote PKB $\alpha$  activity, hatched bars  
19 MAPKAP kinase-1 activity, and grey bars p70 S6 kinase  
20 activity. The activities of each protein kinase are  
21 given relative to their activity towards Crosstide  
22 (100). The results are shown  $\pm$  SEM for two experiments  
23 each carried out in triplicate.

24

#### 25 Discussion.

26 We have established that the minimum consensus sequence  
27 for efficient phosphorylation by PKB $\alpha$  is Arg-Xaa-Arg-  
28 Yaa-Zaa-Ser-Hyd, where Xaa is any amino acid, Yaa and  
29 Zaa are small amino acid other than glycine (Ser, Thr,  
30 Ala) and Hyd is a bulky hydrophobic residue (Phe, Leu)  
31 (Table 7.1). The heptapeptide with the lowest  $K_m$  value  
32 was RPRTSSF, its  $K_m$  of 5  $\mu$ M being comparable to many of  
33 the best peptide substrates identified for other  
34 protein kinases. The  $V_{max}$  for this peptide (250 nmoles  
35 min<sup>-1</sup> mg<sup>-1</sup>) may be an underestimate because the PKB $\alpha$   
36 was obtained by immunoprecipitation from extracts of

1 IGF-1 stimulated 293 cells over-expressing this protein  
2 kinase, and a significant proportion of the PKB $\alpha$  may  
3 not have been activated by IGF-1 treatment.

4  
5 The requirement for arginine residues at positions n-3  
6 and n-5 (where n is the site of phosphorylation) seems  
7 important, because substituting either residue with  
8 lysine decreases phosphorylation drastically. Serine  
9 and threonine residues were preferred at positions n-1  
10 and n-2, although the Km value was only increased about  
11 5-fold if both of these residues were changed to Ala.  
12 Serine was preferred at position n, since changing it  
13 to threonine caused a six-fold increase in the Km.  
14 The peptide RPRAATF, which was phosphorylated with a Km  
15 of 25  $\mu$ M and similar Vmax to RPRTSSF, may therefore be  
16 a better substrate for assaying PKB $\alpha$  in partially  
17 purified preparations, because unlike Crosstide, it  
18 contains only one phosphorylatable residue and is not  
19 phosphorylated significantly by MAPKAP-K1 or p70 S6  
20 kinase (Table 7.2, Fig 18 and see below).

21  
22 The Proline at position n-4 was not altered in this  
23 study because it was already clear that this residue  
24 was not critical for the specificity of PKB $\alpha$ . Residue  
25 n-4 is proline in GSK3 $\beta$  but alanine in GSK3 $\alpha$ . Both GSK3  
26 isoforms are equally good substrates for PKB $\alpha$  in vitro  
27 (Cross et al., 1995), and the peptide  
28 GRARTSSFA (corresponding to the sequence in GSK3 $\alpha$ ) is  
29 phosphorylated by PKB $\alpha$  with a Km of 10  $\mu$ M and Vmax of  
30 230 U/mg (Table 7.1A, peptide 2). Moreover, in histone  
31 H2B, the residue located four amino acids N-terminal to  
32 the PKB $\alpha$  phosphorylation site is serine (Fig 17). The  
33 presence of Glu and Lys at positions n-1 and n-2 may  
34 explain why histone H2B is phosphorylated by PKB $\alpha$  with  
35 a four-fold lower Vmax than the peptide RPRTSSF.

36

1 Two other protein kinases which are activated by  
2 insulin and other growth factors, p70 S6 kinase and  
3 MAPKAP-K1, require basic residues at positions n-3 and  
4 n-5 (Leighton et al., 1995), explaining why they also  
5 phosphorylate and inactivate GSK3 in vitro (Sutherland  
6 et al., 1993). Indeed, there is evidence that MAPKAP-  
7 K1 plays a role in the inhibition of GSK3 by EGF  
8 because, unlike inhibition by insulin which is  
9 prevented by inhibitors of PI 3-kinase, the inhibition  
10 of GSK3 by EGF is only suppressed partially by  
11 inhibitors of PI 3-kinase. Moreover, in NIH 3T3 cells,  
12 the inhibition of GSK3 $\alpha$  and GSK3 $\beta$  by EGF is largely  
13 prevented by expression of a dominant negative mutant  
14 of MAP kinase kinase-1 (Eldar et al., 1995). In  
15 contrast, p70 S6 kinase is not rate limiting for the  
16 inhibition of GSK3 in the cells that have been examined  
17 so far because rapamycin, which prevents the activation  
18 of p70 S6 kinase by EGF or insulin, has no effect on  
19 the inhibition of GSK3 by these agonists (Cross et al.,  
20 1995 and Saito et al., 1994).

21  
22 Additional similarities between p70 S6 kinase, MAPKAP-  
23 K1 and PKB $\alpha$  include the failure to phosphorylate  
24 peptides containing Pro at position n+1 and dislike of  
25 a lysine at the same position. This suggests that, in  
26 vivo, these kinases are unlikely to phosphorylate the  
27 same residues as MAP kinases (which phosphorylates  
28 Ser/Thr-Pro motifs) or protein kinase C (which prefers  
29 basic residues C-terminal to the site of  
30 phosphorylation). However, the present work has also  
31 revealed significant differences in the specificities  
32 of these enzymes. In particular MAPKAP-K1 and (to a  
33 lesser extent) p70 S6 kinase can tolerate substitution  
34 of the Arg at position n-5 by lysine whereas PKB $\alpha$   
35 cannot (see Table 7.1A, Table 7.2A and (Leighton et  
36 al., 1995)). MAPKAP-K1 and p70 S6 kinase can also

1 tolerate, to some extent, substitution of Arg at  
2 position n-3 by Lys. For example, the peptide  
3 KKRNKTLSVA is phosphorylated by MAPKAP-K1 and p70 S6  
4 kinase with  $K_m$  values of 17 and 34  $\mu M$ , respectively,  
5 as compared to  $K_m$  values of 0.7 and 1.5  $\mu M$  for the  
6 peptide KKRNRTLSVA (Table 7.2A). In contrast, PKB $\alpha$   
7 does not phosphorylate the peptide KKRNKTLSVA (Table  
8 7.2A) or any other peptide that lacks Arg at position  
9 n-3. PKB $\alpha$  and p70 S6 kinase, but not MAPKAP-K1,  
10 phosphorylate Thr as well as Ser (Table 7.1A) and can  
11 phosphorylate peptides lacking any residue at position  
12 n+2 ((Leighton et al., 1995) and Table 7.2A), while  
13 PKB $\alpha$  and MAPKAP-K1, but not p70 S6 kinase, can tolerate  
14 substitution of both the n-1 and n-2 positions of the  
15 peptide RPRTSSF with Ala (Table 7.2B). These  
16 differences explain why the peptide RPRAATF is a  
17 relatively specific substrate for PKB $\alpha$ .

18

19 One of the best peptide substrates for MAPKAP-K1 and  
20 p70 S6 kinase (KKRNRTLSVA) was a poor substrate for  
21 PKB $\alpha$  (Table 7.2, peptide 2), despite the presence of  
22 Arg at positions n-3 and n-5. The presence of Leu at  
23 position n-1 and Val at position n+1 are likely to  
24 explain the high  $K_m$  for phosphorylation, because PKB $\alpha$   
25 prefers a small hydrophilic residue at the former  
26 position and a larger hydrophobic residue at the latter  
27 position (Tables 7.1 and 7.2).

28

29 Example 9:

30 This example demonstrates that coexpression of GSK3 in  
31 293 cells with either the wild type or a constitutively  
32 activated PKB results in GSK3 becoming phosphorylated  
33 and inactivated. However coexpression of a mutant of  
34 GSK3 in which Ser-9 is mutated to an Ala residue is not  
35 inactivated under these conditions. These experiments  
36 provide further evidence that PKB $\alpha$  activation can

1 mediate the phosphorylation and inactivation of GSK3 in  
2 a cellular environment, and could be used as an assay  
3 system to search for specific PKB inhibitors.

4  
5 Monoclonal antibodies recognising the sequence EFMPME  
6 (EE) antibodies and the (EQKLISEEDL) c-Myc purchased  
7 from Boehringer (Lewis, UK).

8  
9 Construction of expression vectors and transfections  
10 into 293 cells. HA-PKBa, HA-KD-PKB and 308D/473D  
11 HA-PKBa was described previously (Alessi et al., 1996).

12  
13 A DNA construct expressing human GSK3B with the EFMPME  
14 (EE) epitope tag at the N-terminus was prepared as  
15 follows: A standard PCR reaction was carried out using  
16 as a template the human GSK3 $\beta$  cDNA clone in the  
17 pBluescript SK+ vector and the oligonucleotides

18  
19 GCGGAGATCTGCCACCATGGAGTTCATGCCCATGGAGTCAGGGCGGCCCAAGAAC

20  
21 and GCGGTCCGGAACATAGTCCAGCACCAG that incorporate a *bgl*  
22 II site (underlined) and a *Bspe* I site (double  
23 underlined). A three-way ligation was then set up in  
24 which the resulting PCR product was subcloned as a *Bgl*  
25 II-*Bspe* I fragment together with the C-terminal *Bspe*  
26 I-Cla I fragment of GSK3 $\beta$  into the *Bgl* II-Cla I sites  
27 of the pCMV5 vector (Anderson et al., 1989). The  
28 construct was verified by DNA sequencing and purified  
29 using the Quiagen plasmid Mega kit according to the  
30 manufacturers protocol. The c-Myc GSK3, BA9 construct  
31 encodes GSK3 $\beta$  in which Ser-9 is mutated to Ala and  
32 possesses a c-myc epitope tag at the C-terminus and was  
33 prepared as described in Sperber et al., 1995. The  
34 c-Myc GSK3 $\beta$  A9 gene was then subcloned into *xba* I/ECOR  
35 I sites of the pCMV5 eukaryotic expression vector.

36



1     **Cotransfection of GSK3 $\beta$  with PKBa and its assay.**  
2     293 cells growing on 10 cm diameter dishes were  
3     transfected with 10 ug of DNA constructs expressing  
4     EE-GSK3, Myc-GSK3A9 in the presence or absence of  
5     HA-PKB, HA-KD-PKB or HA-308D/473D-PKB exactly as  
6     described in Alessi et al., 1996. The cells were grown  
7     in the absence of serum for 16 h prior to lysis, and  
8     then lysed in 1.0 ml of ice-cold Buffer A (50 mM  
9     Tris/HCl pH 7.5, 1 mM EDTA 1 mM EGTA, 1% (by vol) Triton  
10    X100, 1 mM sodium orthovanadate, 10 mM sodium  
11    glycerophosphate, 50 mM NaF, 5 mM sodium pyrophosphate,  
12    1uM Microcystin-LR, 0.27 M sucrose, 1 mM benzamidine,  
13    0.2 mM phenylmethylsulphonyl fluoride, 10 ug/ml  
14    leupeptin, and 0.1% (by vol) 2-mercaptoethanol ). The  
15    lysate was centrifuged at 4°C for 10 min at 13, 000 x g  
16    and an aliquot of the supernatant (100 ug protein) was  
17    incubated for 30 min on a shaking platform with 5 ul of  
18    protein G-Sepharose coupled to 1ug of EE monoclonal  
19    antibody. The suspension was centrifuged for 1min at  
20    13,000 x g, the Protein G-Sepharose-antibody-EE-GSK3 $\beta$   
21    complex washed twice with 1.0 ml of Buffer A containing  
22    0.5 M NaCl, and three times with Buffer B ( 50 mM Tris  
23    pH 7.5, 0.1 mM EGTA, 0.01% (by vol) Brij-35 and 0.1%  
24    (by vol) 2-mercaptoethanol ), and the immunoprecipitate  
25    assayed for GSK3 activity after incubation with either  
26    PP2A or microcystin inactivated PP2A as described  
27    previously (Cross et al., 1994).

28

## 29     **Results**

30

31     **Cotransfection of GSK3 $\beta$  with PKBa in 293 cells results**  
32     **in GSK3 phosphorylation and inactivation**  
33     Human embryonic kidney 293 cells were transfected with  
34     a DNA construct expressing EE-epitope tagged GSK3 $\beta$   
35     either in the presence or absence of DNA constructs  
36     expressing wild type-PKBa, a catalytically inactive

1 PKBa or a constitutively active HA-(308D/473D)-PKBa.  
2 Cells were serum starved for 16 h. 36h post  
3 transfection the cells were lysed, and the GSK3 $\beta$   
4 immunoprecipitated from the lysates using monoclonal EE  
5 antibodies and the GSK3 $\beta$  activity measured before and  
6 after treatment with PP2A. When EEGSK3 $\beta$  was expressed  
7 alone or in the presence of a catalytically inactive  
8 PKBa, treatment of the EE-GSK3 $\beta$  with PP2A only resulted  
9 in about a 12% increase in activity (Fig 19A). However  
10 when EE-GSK3 $\beta$  was coexpressed with either the wild type  
11 PKBa or the constitutively activated 308D/473D-HA-PKBa,  
12 treatment of the EE-GSK3 from these cell lysates with  
13 PP2A resulted in a 68% and 85% increase in the GSK3  
14 activity, respectively. Coexpression of Myc-GSK3 $\beta$  A9  
15 with HA-PKB or the constitutively active  
16 308D/473D-HA-PKBa did not result in any significant  
17 inactivation of this mutant of GSK3 as judged by its  
18 ability to be reactivated by PP2A (Fig 19B). These data  
19 demonstrate that even in a cellular environment, PKBa  
20 is capable of phosphorylating GSK3 $\beta$  at Ser-9 and  
21 inactivation of the enzyme. To estimate the relative  
22 levels of EE-GSK3 $\beta$  and PKBa, EE-GSK3 and HA-PKBa were  
23 immunoprecipitated from equal volumes of cell lysate,  
24 and the immunoprecipitates run on an SDS-polyacrylamide  
25 gel, and the gel stained with Coomassie Blue. These  
26 experiments revealed that both the wild type HA-PKBa  
27 and the 308D/473D-PKBa were expressed at a 20 to 30  
28 -fold higher level than GSK3a, whereas KD-PKBa is  
29 expressed at a level that is about 5-fold lower than  
30 that of the wild type PKBa. Under the conditions used  
31 for the immunoprecipitations, no PKBa was  
32 co-immunoprecipitated with GSK3 $\beta$ , or no GSK3 $\beta$  was  
33 co-immunoprecipitated with the PKBa (data not shown).  
34 Coexpression of EE-GSK3 $\beta$  with all forms of PKBa  
35 resulted in about a 2-3 fold decrease in the level of  
36 expression on EE-GSK3 $\beta$  compared to when it is expressed

1 alone in cells.

2

3 Example 10: basic assay for identifying agents which  
4 affect the activity of PKB.

5 A 40  $\mu$ l assay mix was prepared containing protein  
6 kinase (0.2U/ml) in 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA,  
7 0.1% (by vol) 2-mercaptoethanol, 2.5  $\mu$ M PKI, protein  
8 kinase substrate (30 $\mu$ M), and the indicated  
9 concentration of Ro-318220 or GC 109203X (test  
10 inhibitors). After incubation on ice for 10 min the  
11 reaction was started by the addition of 10  $\mu$ l of 50mM  
12 magnesium acetate and 0.5 mM [ $\gamma$ <sup>32</sup>P]ATP (100-200  
13 cpm/pmol). For the assay of mixed isoforms of PKC 20  
14  $\mu$ M diacylglycerol, 0.5 mM CaCl<sub>2</sub>, and 100  $\mu$ M  
15 phosphatidylserine were also present in the  
16 incubations. The assays were carried out for 15 min at  
17 30°C, then terminated and analysed as described (Alessi  
18 1995). One unit of activity was that amount of enzyme  
19 that catalysed the phosphorylation of 1nmol of  
20 substrate in 1 min. The final concentration of DMSO in  
21 each assay was 1% (by vol). This concentration of DMSO  
22 does not inhibit any of these enzymes. Mixed isoforms  
23 of PKC were assayed using histone H1 as substrate,  
24 while MAPKAP-K1 $\beta$  and p70 S6 kinase were assayed using  
25 the peptide KKRNRTLSVA (Leighton 1995). Protein kinase  
26 B was assayed with the peptide GRPRTSSFAEG [9] and  
27 MAPKAP-K2 was assayed with the peptide KKLNRTLSVA  
28 (Stokoe 1993). p42 MAP kinase was assayed using MBP,  
29 and MAPKK-1, and c-Raf1 were assayed as described in  
30 Alessi 1995.

31

### 32 Results

33 Effect of Ro 318220 and GF 109203X on protein kinases  
34 activated by growth factors, cytokines and cellular  
35 stresses. The mixed isoforms of PKC were potently  
36 inhibited by Ro 318220, with an IC<sub>50</sub> of 5 nM in our

1 assay (Fig 20A). In contrast, a number of protein  
2 kinases activated by growth factors (c-Raf1, MAPKK-1,  
3 p42 MAP kinase) and one protein kinase that is  
4 activated by cellular stresses and proinflammatory  
5 cytokines (MAPKAP-K2) were not inhibited significantly  
6 by Ro 318022 in vitro (Fig 20A). Protein kinase B, an  
7 enzyme that is activated in response to insulin and  
8 growth factors was inhibited by Ro 318220 ( $IC_{50}$  of 1  $\mu$ M,  
9 Fig 20B) similar to the  $IC_{50}$  for PKA. However, to our  
10 surprise, MAPKAP-K1B an enzyme which lies immediately  
11 downstream of p42 and p44 MAP kinases and which is  
12 activated in response to every agonist that stimulates  
13 this pathway, was inhibited by Ro 318220 even more  
14 potently than the mixed PKC isoforms ( $IC_{50}$  = 3nM, Fig  
15 20B). The p70 S6 kinase, which lies on a distinct  
16 growth factor-stimulated signalling pathway from  
17 MAPKAP-K1B, was also potently inhibited by Ro 318220  
18 ( $IC_{50}$ =15 nM, Fig 20B).

19  
20 Similar results were obtained using GF 109203X instead  
21 of Ro 3318220. As reported previously (Toullec et al.,  
22 1991), GC 109203X inhibited the mixed isoforms of PKC  
23 ( $IC_{50}$ =30 nM) without inhibiting protein kinase B (Fig  
24 21) or c-Raf, MAPKK-1 and p42 MAP kinase (data not  
25 shown). However MAPKAP-K1B and p70 S6 kinase were  
26 potently inhibited by this compound with  $IC_{50}$  values of  
27 50 nM and 100 nM, respectively (Fig 21).

28  
29

1     General Materials and Methods Tissue culture reagents, myelin  
2     basic protein (MBP), microcystin-LR, and IGF-1 were obtained from  
3     Life Technologies Inc. (Paisley, UK), insulin from Novo-Nordisk  
4     (Bagsvaerd, Denmark), phosphate free Dulbecco's minimal essential  
5     medium (DMEM) from (ICN, Oxon, UK), Protein G-Sepharose and  
6     CH-Sepharose from Pharmacia (Milton Keynes, UK), alkylated trypsin  
7     from Promega (Southampton, UK), 4-vinylpyridine, wortmannin and  
8     fluoroisothiocyanate-labelled antimouse IgG from goat from  
9     Sigma-Aldrich (Poole, Dorset, UK). Polyclonal antibodies were  
10    raised in sheep against the peptides RPHFPQFSYSASGTA  
11    (corresponding to the last 15 residues of rodent PKB $\alpha$ ) and  
12    MTSALATMRVDYEQIK (corresponding to residues 352 to 367 of human  
13    MAPKAPkinase-2) and affinity purified on peptide-CH-Sepharose.  
14    Monoclonal HA antibodies were purified from the tissue culture  
15    medium of 12CA5 hybridoma and purified by chromatography on  
16    Protein G-Sepharose. The peptide RPRHFPQFSYSAS, corresponding to  
17    residues 465-478 of PKB $\alpha$ , was synthesized on an Applied Biosystems  
18    430A peptide synthesizer. cDNA encoding residues 46-400 of human  
19    MAPKAP kinase-2 was expressed in E.coli as a glutathione  
20    S-transferase fusion protein and activated with p38/RK MAP KINASE  
21    by Mr A.Clifton (University of Dundee) as described previously  
22    (Ben-Levy et al., 1995).

23  
24    Monoclonal antibodies recognising the haemagglutinin (HA) epitope  
25    sequence YPYDVPDYA, Protein G-Sepharose and histone H2B were  
26    obtained from Boehringer (Lewes, UK). MAPKAP kinase-1 (Sutherland  
27    et al., 1993) and p70 S6 kinases (Leighton et al., 1995) were  
28    purified from rabbit skeletal muscle and rat liver respectively.

29  
30    Construction of expression vectors. The pECE constructs encoding  
31    the human HAPKB $\alpha$  and kinase-dead (K179A) HA-KD-PKB $\alpha$  have already  
32    been described (Andjelkovic et al., 1996). The mutants at Ser-473  
33    (HA-473A PKB $\alpha$  and HA-473D PKB $\alpha$  were created by PCR using a 5'  
34    oligonucleotide encoding amino acids 406 - 414 and mutating 3'  
35    oligonucleotide encoding amino acids 468 - 480, and the resulting  
36    PCR products subcloned as *CelIII-EcoRI* fragment into pECE.HA-PKB $\alpha$ .  
37    The Thr-308 mutants (HA-308A PKB $\alpha$  and HA308D PKB $\alpha$ ) were created by  
38    the two-stage PCR technique (No et al., 1989) and subcloned as  
39    *NotI-EcoRI* fragments into pECE.HA-PKB. The double mutant  
40    HA-308D/473D PKB was made by subcloning the *CelIII-EcoRI* fragment  
41    encoding 473D into pECE.HA-308D PKB $\alpha$ . For construction of  
42    cytomegalovirus-driven expression constructs, *BglII-XbaI* fragments  
43    from the appropriate pECE constructs were subcloned into the same  
44    restriction sites of the pCMV5 vector (Andersson et al., 1989).

1 All constructs were confirmed by restriction analysis and  
2 sequencing and purified using Quiagen Plasmid Maxi Kit according  
3 to the manufacturer's protocol. All oligonucleotide sequences are  
4 available upon request.

5  
6 **<sup>32</sup>P-labelling of L6 myotubes and immunoprecipitation of PKB $\alpha$ .** L6  
7 cells were differentiated into myotubes on 10 cm diameter dishes  
8 (Hundal et al., 1992). The myotubes were deprived of serum  
9 overnight in DMEM, washed three times in phosphate free DMEM and  
10 incubated for a further 1 h with 5 ml of this medium. The myotubes  
11 were then washed twice with phosphate free DMEM and incubated for  
12 4 h with carrier-free [<sup>32</sup>P]orthophosphate (1 mCi/ml). Following  
13 incubation in the presence or absence of 100 nM wortmannin for 10  
14 min, the myotubes were stimulated for 5 min at 37°C in the  
15 presence or absence of 100 nM insulin and placed on ice. The  
16 medium was aspirated, the myotubes washed twice with ice-cold DMEM  
17 buffer and then lysed with 1.0 ml of ice-cold Buffer A (50 mM  
18 Tris/HCl pH 7.5, 1 mM EDTA 1 mM EGTA, 1% (by vol) Triton X100, 1 mM  
19 sodium orthopervanadate, 10 mM sodium glycerophosphate, 50 mM NaF,  
20 5 mM sodium pyrophosphate, 1  $\mu$ M Microcystin-LR, 0.27 M sucrose, 1  
21 mM benzamidine, 0.2 mM phenylmethylsulphonyl fluoride, 10  $\mu$ g/ml  
22 leupeptin, and 0.1% (by vol) 2-mercaptoethanol). The lysates were  
23 centrifuged at 4°C for 10 min at 13,000 x g and the supernatants  
24 incubated for 30 min on a shaking platform with 50  $\mu$ l of Protein  
25 G-Sepharose coupled to 50  $\mu$ g of preimmune sheep IgG. The  
26 suspensions were centrifuged for 2 min at 13,000 x g and the  
27 supernatants incubated for 60 min with 30  $\mu$ l of Protein G--  
28 Sepharose covalently coupled to 60  $\mu$ g of PKB $\alpha$  antibody (Harlow and  
29 Lane, 1988). The Protein G-Sepharose-antibody-PKB $\alpha$  complex was  
30 washed eight times with 1.0 ml of Buffer A containing 0.5 M NaCl,  
31 and twice with 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA and 0.1% (by  
32 vol) 2-mercaptoethanol (Buffer B).

33  
34 **Assay of immunoprecipitated PKB $\alpha$  and protein determinations.** Three  
35 aliquots of each immunoprecipitate (each comprising only 5% of the  
36 total immunoprecipitated PKB $\alpha$ ) were assayed for PKB $\alpha$  activity  
37 towards the peptide GRPRTSSFAEG as described previously (Cross et  
38 al., 1995). One unit of activity was that amount which catalysed  
39 the phosphorylation of 1 nmol of substrate in 1 min. Protein  
40 concentrations were determined by the method of Bradford, 1976.

41  
42 **Tryptic digestion of in vivo phosphorylated PKB $\alpha$ .** The  
43 immunoprecipitated PKB $\alpha$  was added to an equal volume of 2% (by

1 mass) SDS and 2 % (by vol) 2-mercaptoethanol, and incubated for 5  
2 min at 100°C. After cooling to room temperature, 4-vinylpyridine  
3 was added to a final concentration of 2 % (by vol) and the mixture  
4 was incubated for 1h at 30°C on a shaking platform, followed by  
5 electrophoresis on a 10% polyacrylamide gel. After  
6 autoradiography, the 60 kDa band corresponding to rat PKB $\alpha$  was  
7 excised and the gel piece homogenized in five vols of 25 mM  
8 N-ethylmorpholine HCl, pH 7.7, containing 0.1% (by mass) SDS and 5  
9 % (by vol) 2-mercaptoethanol. The suspension was incubated for 1 h  
10 at 37°C on a shaking platform, then centrifuged for 1 min at  
11 13,000 x g and the supernatant collected. The pellet was incubated  
12 for a further 1h with five vols of the same buffer and centrifuged  
13 for 1min at 13,000 xg. The two supernatants (containing 80-90% of  
14 the <sup>32</sup>P-radioactivity) were combined, 0.2 vols of 100% (by mass)  
15 trichloroacetic acid added, and the sample incubated for 1 h on  
16 ice. The suspension was centrifuged for 10 min at 13,000 x g, the  
17 supernatant discarded and the pellet washed five times with 0.2 ml  
18 of water. The pellet was then incubated at 30°C with 0.3 ml of 50  
19 mM Tris/HCl pH 8.0, 0.1% (by vol) Triton X100 containing 1 $\mu$ g of  
20 alkylated trypsin. After 3 h another 1 $\mu$ g of trypsin was added and  
21 the suspension left for a further 12 h. Guanidinium hydrochloride  
22 (8 M) was added to bring the final concentration to 1.0 M in order  
23 to precipitate any residual SDS and, after standing on ice for 10  
24 min, the suspension was centrifuged for 5 min at 13, 000 x g. The  
25 supernatant containing 90 % of the <sup>32</sup>P-radioactivity was  
26 chromatographed on a Vydac C18 column as described in the legend  
27 to Fig 2.

28  
29 **Transfection of 293 cells and immunoprecipitation of HA-tagged**  
30 **PKB $\alpha$ .** Human embryonic kidney 293 cells were cultured at 37°C in  
31 an atmosphere of 5% CO<sub>2</sub>, on 10 cm diameter dishes in DMEM  
32 containing 10 % foetal calf serum. Cells were split to a density  
33 of 2 x 10<sup>6</sup> per 10 cm dish, and after 24 h at 37°C the medium was  
34 aspirated and 10 ml of freshly prepared DMEM containing 10 %  
35 foetal calf serum added. Cells were transfected by a modified  
36 calcium phosphate method (Chen and Okayama, 1988) with 1 $\mu$ g/ml DNA  
37 per plate. 10  $\mu$ g of plasmid DNA in 0.45 ml of sterile water was  
38 added to 50  $\mu$ l of sterile 2.5 M CaCl<sub>2</sub>, and then 0.5 ml of a  
39 sterile buffer composed of 50 mM N,N-bis[2-hydroxyethyl]-2-  
40 aminoethanesulphonic acid/HCl pH 6.96, 0.28 M NaCl and 1.5 mM  
41 Na<sub>2</sub>HPO<sub>4</sub> was added. The resulting mixture was vortexed for 1 min,  
42 allowed to stand at room temperature for 20 min, and then added  
43 dropwise to a 10 cm dish of 293 cells). The cells were placed in

1 an atmosphere of 3% CO<sub>2</sub>, for 16 h at 37°C, then the medium was  
2 aspirated, and replaced with fresh DMEM containing 10% foetal calf  
3 serum. The cells were incubated for 12 h at 37°C in an atmosphere  
4 of 5% CO<sub>2</sub>, and then for 12 h in DMEM in the absence of serum.  
5 Cells were preincubated for 10 min in the presence of 0.1% DMSO or  
6 100 nM wortmannin in 0.1% DMSO and then stimulated for 10 min with  
7 either 100 nM insulin or 50 ng/ml IGF-1 in the continued presence  
8 of wortmannin. After washing twice with ice cold DMEM the cells  
9 were lysed in 1.0 ml of icecold Buffer A, the lysate was  
10 centrifuged at 4°C for 10 min at 13,000 x g and an aliquot of the  
11 supernatant (10 µg protein) was incubated for 60 min on a shaking  
12 platform with 5 µl of protein G-Sepharose coupled to 2 µg of HA  
13 monoclonal antibody. The suspension was centrifuged for 1 min at  
14 13,000 x g, the Protein G-Sepharose-antibody-HA-PKBα complex  
15 washed twice with 1.0 ml of Buffer A containing 0.5 M NaCl, and  
16 twice with Buffer B, and the immunoprecipitate assayed for PKBα  
17 activity as described above.

18  
19 <sup>32</sup>P-Labeling of 293 cells transfected with HA-PKBα. 293 cells  
20 transfected with HA-PKBα DNA constructs. were washed with  
21 phosphate free DMEM, incubated with [32p] orthophosphate (1  
22 mCi/ml) as described for L6 myotubes, then stimulated with insulin  
23 or IGF1 and lysed, and PKBα immunoprecipitated as described above.  
24 The <sup>32</sup>P-labelled HA-PKBα immunoprecipitates were washed, alkylated  
25 with 4-vinylpyridine, electrophoresed and digested with trypsin as  
26 described above for the endogenous PKBα present in rat L6  
27 myotubes.

28  
29 Transfection of COS-1 cells and immunoprecipitation of HA-PKBα.  
30 COS-1 cells were maintained in DMEM supplemented with 10% FCS at  
31 37°C in an atmosphere of 5% CO<sub>2</sub>. Cells at 70 - 80% confluency  
32 were transfected by a DEAE-dextran method (Seed & Aruffo, 1987),  
33 and 48 hours later serum-starved for 24 hours. Cells were lysed in  
34 a buffer containing 50 mM Tris-HCl, pH 7.5, 120 mM NaCl, 1% Nonidet  
35 P-40, 25 mM NaF, 40 mM sodium-β-glycerophosphate, 0.1 mM sodium  
36 orthopervanadate, 1 mM EDTA, 1mM benzamidine, 1 mM  
37 phenylmethylsulphonyl fluoride, and lysates centrifuged for 15 min  
38 at 13,000 x g at 4°C. Supernatants were pre cleared once for 30  
39 min at 4°C with 0.1 vols of 50% Sepharose 4B/25% Pansorbin  
40 (Pharmacia and Calbiochem, respectively) and HA-PKBα  
41 immunoprecipitated from 1 mg of extract using the 12CA5 antibody  
42 coupled to Protein A Sepharose beads. Immunoprecipitates were  
43 washed twice with lysis buffer containing 0.5 M NaCl and once with



1 lysis buffer.

2

3 **Immunoblotting and quantification of levels of PKB $\alpha$  expression.**

4 Cell extracts were resolved by 7.5% SDS-PAGE and transferred to

5 Immobilon membranes (Millipore). Filters were blocked for 30 min

6 in a blocking buffer containing 5% skimmed milk in 1x TBS, 1%

7 Triton X-100 and 0.5% Tween 20, followed by a 2h incubation with

8 the 12CA5 supernatant 1000-fold diluted in the same buffer. The

9 secondary antibody was alkaline conjugated anti-mouse Ig from goat

10 (Southern Biotechnology Associates, Inc), 1000-fold diluted in the

11 blocking buffer. Detection was performed using AP colour

12 development reagents from Bio-Rad according to the manufacturer's

13 instructions. Quantification of levels of PKB $\alpha$  expression was

14 achieved by chemiluminescence, using fluoroisothiocyanate-labelled

15 antimouse IgG from goat as the secondary antibody and the Storm

16 840/860 and ImageQuant software from Molecular Dynamics.

17

18 All peptides used to assay PKB $\alpha$ , and TTYADFIASGRTGRRNAIHD (the

19 specific peptide inhibitor of cyclic AMP dependent protein kinase

20 - PKI) were synthesised on an Applied Biosystems 431A peptide

21 synthesizer. Their purity (> 95%) was established by HPLC and

22 electrospray mass spectrometry, and their concentrations were

23 determined by quantitative amino acid analysis.

24

25 **Preparation and assay of PKB $\alpha$ .** The construction of cytomegalovirus

26 vectors (pCMV5) of the human haemagglutinin epitope-tagged wild

27 type (HA-PKB $\alpha$ ) was described previously (Alessi et al., 1996).

28 293 cells grown on 10 cm dishes were transfected with a DNA

29 construct expressing HA-PKB $\alpha$  using a modified calcium phosphate

30 procedure (Alessi et al., 1996). The cells were deprived of serum

31 for 16h prior to lysis and, where indicated, were stimulated for

32 10 min in the presence of 50 ng/ml IGF-1 to activate PKB $\alpha$ . The

33 cells were lysed in 1.0 ml ice-cold Buffer A (50 mM Tris/HCl pH

34 7.5, 1 mM EDTA 1 mM EGTA, 1% (by vol) Triton X-100, 1 mM sodium

35 orthovanadate, 10 mM sodium  $\beta$ -glycerophosphate, 50 mM NaF, 5 mM

36 sodium pyrophosphate, 1  $\mu$ M Microcystin-LR, 0.27 M sucrose, 1 mM

37 benzamidine, 0.2 mM phenylmethylsulphonyl fluoride, 10  $\mu$ g/ml

38 leupeptin, and 0.1 % (by vol) 2-mercaptoethanol) the lysate

39 centrifuged at 4°C for 10 min at 13, 000 x g and the supernatant

40 obtained from one 10 cm dish of cells (2-3 mg protein) was

41 incubated for 60 min on a shaking platform with 20  $\mu$ l of protein

42 G-Sepharose coupled to 10  $\mu$ g of HA monoclonal antibody. The

43 suspension was centrifuged for 1 min at 13, 000 x g, the Protein

1 G-Sepharose-antibody-HA-PKB $\alpha$  complex washed twice with 1.0 ml of  
2 Buffer A containing 0.5 M NaCl, and twice with Buffer B (50 mM  
3 Tris/HCl pH 7.5, 0.1 mM EGTA, 0.01% (by vol) Brij-35 and 0.1% (by  
4 vol) 2-mercaptoethanol). The PKB $\alpha$  immunoprecipitates were diluted  
5 in Buffer B to an activity of 2.0 U/ml towards the Crosstide  
6 peptide GRPRTSSFAEG and 0.1 ml aliquots snap frozen in liquid  
7 nitrogen and stored at -80 oC. No significant loss of PKB $\alpha$   
8 activity occurred upon thawing the PKB $\alpha$  immunoprecipitates or  
9 during storage at -80oC for up to 3 months . The standard  
10 PKB $\alpha$ \_assay (50  $\mu$ l) contained: 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA,  
11 0.1% (by vol) 2-mercaptoethanol, 2.5  $\mu$ M PKI, 0.2 U/ml PKB $\alpha$ ,  
12 Crosstide (30  $\mu$ M), 10 mM magnesium acetate and 0.1 mM [ $\gamma$ <sup>32</sup>P]ATP  
13 (100-200 cpm/pmol). The assays were carried out for 15 min at  
14 30oC, the assay tubes being agitated continuously to keep the  
15 immunoprecipitate in suspension, then terminated and analysed as  
16 described (Alessi et al., 1995). One unit of activity was that  
17 amount of enzyme which catalysed the phosphorylation of 1 nmol of  
18 Crosstide in 1 min. The phosphorylation of other peptides, histone  
19 H2B and MBP were carried out in an identical manner. All the  
20 Crosstide activity in HA-PKB $\alpha$  immunoprecipitates is catalysed by  
21 PKB $\alpha$  (see Results) and the PKB $\alpha$  concentration in the  
22 immunoprecipitates was estimated by densitometric scanning of  
23 Coomassie blue-stained polyacrylamide gels, using bovine serum  
24 albumin as a standard. Protein concentrations were determined by  
25 the method of Bradford using bovine serum albumin as standard  
26 (Bradford et al., 1976). Michaelis constants (Km) and Vmax values  
27 were determined from double reciprocal plots of 1/V against 1/S,  
28 where V is the initial rate of phosphorylation, and S is the  
29 substrate concentration. The standard errors for all reported  
30 kinetic constants were within  $\pm$  20%, and the data is reported as  
31 mean values for 3 independent determinations. Fig 16 shows the  
32 results relative to those obtained for unstimulated PKB $\alpha$ .

33  
34 **Tryptic digestion of histone 2B phosphorylated by PKB $\alpha$ .** Histone  
35 H2B (30  $\mu$ M) was phosphorylated with 0.2 U/ml HA-PKB $\alpha$ . After 60 min  
36 0.2 vol of 100% (by mass) trichloroacetic acid was added, and the  
37 sample incubated for 1 h on ice. The suspension was centrifuged  
38 for 10 min at 13, 000 x g, the supernatant discarded and the  
39 pellet washed five times with 0.2 ml of ice cold acetone. The  
40 pellet was resuspended in 0.3 ml of 50 mM Tris/HCl pH 8.0, 0.1%  
41 (by vol) reduced Triton-X100 containing 2  $\mu$ g of alkylated trypsin  
42 and, after incubation for 16 h at 30oC, the digest was centrifuged  
43 for 5 min at 13, 000 x g. The supernatant, containing 95% of the

1     <sup>32</sup>P-radioactivity, was chromatographed on a Vydac C18 column  
2     equilibrated with 0.1% (by vol) trifluoroacetic acid (TFA) in  
3     water. With reference to the results shown in Fig 17, the columns  
4     were developed with a linear acetonitrile gradient (diagonal line)  
5     at a flow rate of 0.8 ml / min and fractions of 0.4 ml were  
6     collected. (A) Tryptic peptide map of <sup>32</sup>P-labelled histone H2B,  
7     70% of the radioactivity applied to the column was recovered from  
8     the major <sup>32</sup>P-peptide eluting at 19.5% acetonitrile. (B) A portion  
9     of the major <sup>32</sup>P-peptide (50 pmol) was analysed on an Applied  
10    Biosystems 476A sequencer, and the phenylthiohydantoin (Pth) amino  
11    acids identified after each cycle of Edman degradation are shown  
12    using the single letter code for amino acids. A portion of the  
13    major <sup>32</sup>P-peptide (1000 cpm) was then coupled covalently to a  
14    Sequelon arylamine membrane and analysed on an Applied Biosystems  
15    470A sequencer using the modified programme described in (Stokoe  
16    et al., 1992). <sup>32</sup>P radioactivity was measured after each cycle of  
17    Edman degradation.  
18  
19

Table 7.1

**Molecular basis for the substrate specificity of PKB $\alpha$** 

The phosphorylated residue is shown in boldface type, the altered residue is underlined. V(100  $\mu$ M) is the relative rate of phosphorylation at 0.1 mM peptide relative to peptide 1. ND, not determined. \*An alanine residue was added to the C-terminal of the peptide RPRTSSP, since we have experienced difficulty in synthesizing peptides terminating in proline.

A	Peptides	K <sub>m</sub> ( $\mu$ M)	V <sub>max</sub> (U/mg)	V(0.1 mM)
1.	GR <b>P</b> RTSSFAEG	4	250	100
2.	RPRTSSFA	8	305	109
3.	GR <b>P</b> RTSSF	8	385	129
4.	RPRTSSF	5	260	105
5.	RPRT <b>S</b> TF	30	243	78
6.	RPRT <b>S</b> AF	-	0	
7.	PRTSSF	-	0	
8.	RPRTSS	>500	ND	2
9.	<b>K</b> PRTSSF	>500	ND	4
10.	RP <b>K</b> TSSF	>500	ND	2
<b>B</b>				
1.	RPRTSSF	5	260	105
2.	RPRTSS <b>L</b>	8	278	104
3.	RPRTSS <b>V</b>	21	300	102
4.	RPRTSS <b>A</b>	250	265	30
5.	RPRTSS <b>K</b>	80	308	67
6.	RPRTSS <b>E</b>	>500	ND	9
7.	RPRTSS <b>P</b> A*	-	0	
<b>C</b>				
1.	RPRTSSF	5	260	105
2.	RPR <b>A</b> SSF	12	230	89
3.	RPR <b>V</b> SSF	25	273	77
4.	RPR <b>G</b> SSF	60	163	37
5.	RPR <b>N</b> SSF	>500	ND	21
6.	RPRT <b>A</b> SF	20	213	83
7.	RPRT <b>G</b> SF	25	233	77
8.	RPRT <b>V</b> SF	30	365	89
9.	RPRT <b>N</b> SF	30	300	81
10.	RPRA <b>A</b> SF	25	215	77
11.	RPR <b>G</b> GSF	105	345	55
12.	RPR <b>G</b> ASF	105	160	37
13.	RPR <b>A</b> GSF	49	114	70

**Table 7.2 Comparison of the substrate specificities of PKB $\alpha$ , MAPKAP kinase-1, and p70S6 kinase.**  
 Peptides 1 and 2 are very good substrates for MAPKAP kinase-1 and p70 S6 kinase, and peptide 3 is a relatively specific substrate for p70 S6 kinase [16]. \*Data reported previously [16]; ND, not determined.

A	Peptide	Protein kinase B $\alpha$		MAPKAP kinase-1		p70 S6 kinase	
		K <sub>m</sub> (mM)	V <sub>max</sub> (U/mg)	K <sub>m</sub> (mM)	V <sub>max</sub> (U/mg)	K <sub>m</sub> (mM)	V <sub>max</sub> (U/mg)
1.	KKKNRTLVA	185	270	0.2*	1550*	3.3*	890*
2.	KKRNRTLVA	80	300	0.7*	1800*	1.5*	1520*
3.	KKRNKTLVA	>500	ND	17*	840*	34*	760*
4.	KKRNRTLTV	388	330	40*	270*	4.8*	1470*
<b>B</b>							
1.	GRPRTSSFAEG	4	250	2	790	3	1270
2.	RPRTSSF	5	260	12	840	125	705
3.	RPRTSTF	30	240	>500	ND	211	590
4.	RPRAASF	25	215	20	1020	>500	ND
5.	RPRAATF	25	230	>500	ND	>500	ND

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33

1

2 Claims:

3

4 1 The use of a composition of PKB, its analogues,  
5 isoforms, inhibitors, activators and/or the functional  
6 equivalents thereof, to regulate glycogen metabolism  
and/or protein synthesis.

7

8 2 The use of a composition of PKB, its analogues,  
9 isoforms, inhibitors, activators and/or the functional  
10 equivalents thereof, for the manufacture of a  
11 medicament to regulate glycogen metabolism and/or  
12 protein synthesis.

13

14 3 The use as claimed in claim 1 or claim 2, to  
15 combat disease states where glycogen metabolism and/or  
16 protein synthesis exhibits abnormality.

17

18 4 The use as claimed in claim 1, 2 or 3, to combat  
19 diabetes.

20

21 5 The use as claimed in any preceding claim, to  
22 combat cancer.

23

24 6 The use as claimed in claim 5, wherein the cancer  
25 is breast, pancreatic or ovarian cancer.

26

27 7 The use as claimed in any preceding claim, wherein  
28 the PKB is PKB $\alpha$ ,  $\beta$  or  $\gamma$ , an analogue, isoform,  
29 inhibitor, activator or a functional equivalent  
30 thereof.

31

32 8 The use as claimed in any preceding claim, wherein  
33 the PKB, its analogue, isoform, or functional  
34 equivalent is modified at one or both of amino acids  
35 308 and 473 by phosphorylation and/or mutation.

36

1     9     A composition of PKB, its analogues, isoforms,  
2     inhibitors, activators and/or the functional  
3     equivalents thereof.

4  
5     10    A peptide having or including the amino acid  
6     sequence Arg-Xaa-Arg-Yaa-Zaa-Ser/Thr-Hyd, where Xaa is  
7     any amino acid, Yaa and Zaa are any amino acid, and Hyd  
8     is a large hydrophobic residue, or a functional  
9     equivalent of such a peptide.

10

11    11    A peptide as claimed in claim 10, wherein Hyd is  
12    Phe or Leu, or a functional equivalent thereof.

13

14    12    A peptide as claimed in claim 10 or claim 11,  
15    wherein Yaa or Zaa or both are an amino acid other than  
16    glycine.

17

18    13    A peptide as claimed in claim 10, having the amino  
19    acid sequence GRPRTSSFAEG, or a functional equivalent  
20    thereof.

21

22    14    A method of identifying agents able to influence  
23    the activity of GSK3, said method comprising:

24

25    a.    exposing a test substance to a substrate of GSK3;  
26           and

27    b.    detecting whether said substrate has been  
28           phosphorylated.

29

30    15    A method of identifying agents which influence the  
31    activity of PKB, comprising:

32

33    a.    exposing a test substance to a sample containing  
34           PKB, to form a mixture;

35    b.    exposing said mixture to a peptide as claimed in  
36           claim 10, 11, 12 or 13; and

1 c. detecting whether (and, optionally, to what  
2 extent) said peptide has been phosphorylated.  
3

4 16 A method as claimed in claim 14 or 15, wherein the  
5 extent of phosphorylation of the peptide is determined.  
6

7 17 A method as claimed in claim 15, wherein the  
8 phosphorylation state(s) of one or both of amino acids  
9 308 and 473 on PKB is determined.  
10

11 18 A method as claimed in any one of claims 14 to 17,  
12 wherein the test substance is an analogue, isoform,  
13 inhibitor, or activator of PKB.  
14

15 19 A method as claimed in any one of claims 14 to 18,  
16 wherein steps a or b (or both) are carried out in the  
17 presence of divalent cations and ATP.  
18

19 20 A method of treatment of the human or non-human  
20 animal body, said method comprising administering PKB,  
21 its analogues, inhibitors, stimulators or functional  
22 equivalents thereof to said body.  
23

24 21 A method as claimed in claim 20, to combat disease  
25 states where glycogen metabolism and/or protein  
26 synthesis exhibits abnormality.  
27

28 22 A method as claimed in claim 20 or 21, to combat  
29 diabetes.  
30

31 23 A method as claimed in claim 20 or 22, to combat  
32 cancer.  
33

34 24 A method as claimed in claim 23, wherein the  
35 cancer is breast, pancreatic or ovarian cancer.  
36

1     25     A method as claimed in any one of claims 20 to 24,  
2     wherein the PKB is PKB $\alpha$ ,  $\beta$  or  $\gamma$ , an analogue, isoform,  
3     inhibitor, activator or a functional equivalent  
4     thereof.

5

6     26     An agent capable of influencing the activity of  
7     PKB, its isoforms, analogues and/or functional  
8     equivalents, by modifying amino acids 308 and/or 473 by  
9     phosphorylation or mutation.

10

11    27     A method of determining the ability of a substance  
12    to affect the activity or activation of PKB, the method  
13    comprising exposing the substance to PKB and  
14    phosphatidyl inositol polyphosphate and determining the  
15    interaction between PKB and the phosphatidyl inositol  
16    polyphosphate.

17

18    28     A method of determining the ability of a substance  
19    to combat diabetes, cancer, or any disorder which  
20    involves irregularity of protein synthesis or glycogen  
21    metabolism, the method comprising exposing the  
22    substance to PKB and phosphatidyl inositol  
23    polyphosphate and determining the interaction between  
24    PKB and the phosphatidyl inositol polyphosphate.

25

26    29     A method as claimed in claim 27 or claim 28,  
27    wherein the interaction between PKB and the  
28    phosphatidyl inositol polyphosphate is measured by  
29    assessing the phosphorylation state of PKB.

30

31    30     A method as claimed in claim 29, wherein the  
32    phosphorylation state of PKB at T308 and/or S473 is  
33    assessed.

34

35    31     A method of identifying activators or inhibitors  
36    of GSK3 comprising exposing the substance to be tested

1 to GSK3 and determining the state of activation of  
2 GSK3.

3

4 32 A method as claimed in claim 31 wherein the state  
5 of activation of GSK3 is determined by assessing its  
6 phosphorylation.

7

8 33 A method of determining the suitability of a test  
9 substance for use in combatting diabetes, cancer, or  
10 any disorder which involves irregularity of protein  
11 synthesis or glycogen metabolism, the method comprising  
12 exposing the substance to be tested to GSK3 and  
13 determining the state of activation of GSK3.

14

15 34 A method for screening for inhibitors or  
16 activators of enzymes that catalyse the phosphorylation  
17 of PKB, the method comprising exposing the substance to  
18 be tested to

19 - one or more enzymes upstream of PKB;

20 - PKB; and (optionally)

21 - nucleoside triphosphate

22 and determining whether (and optionally to what extent)

23 the PKB has been phosphorylated on T308 and/or S473.

1/28

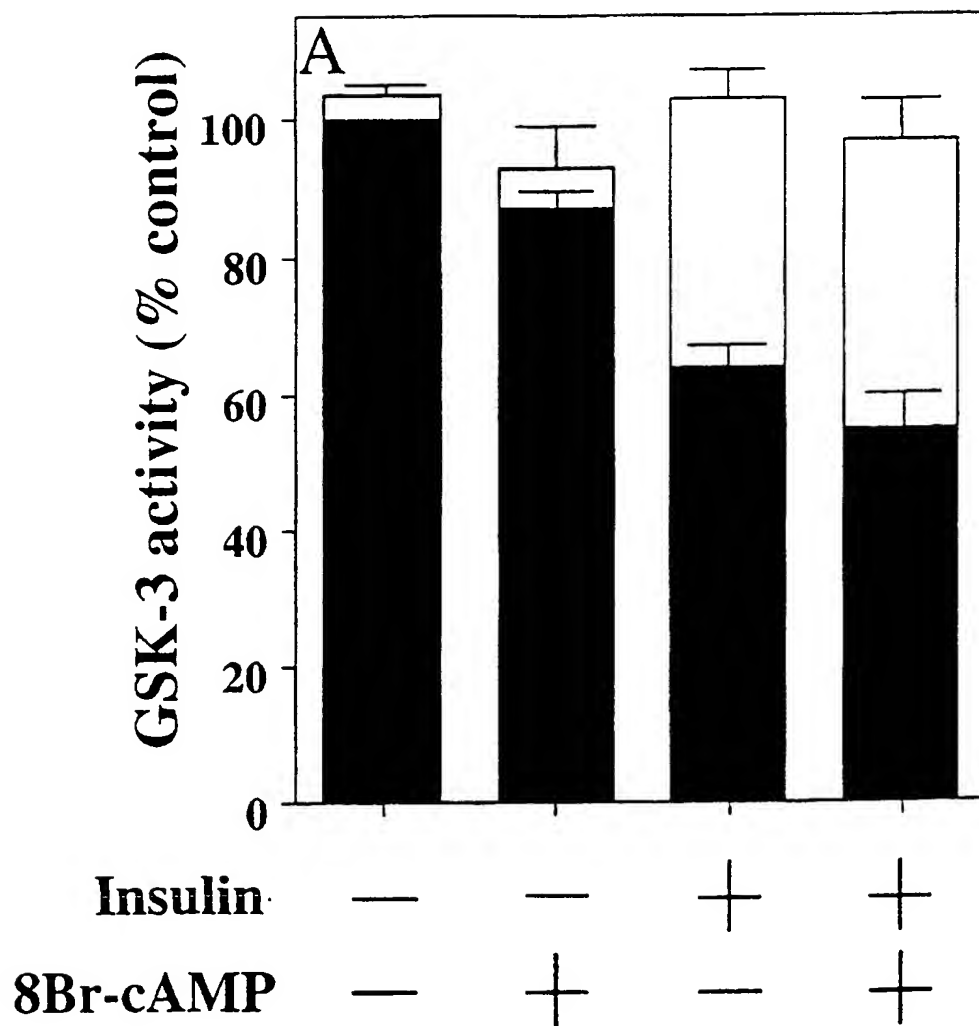
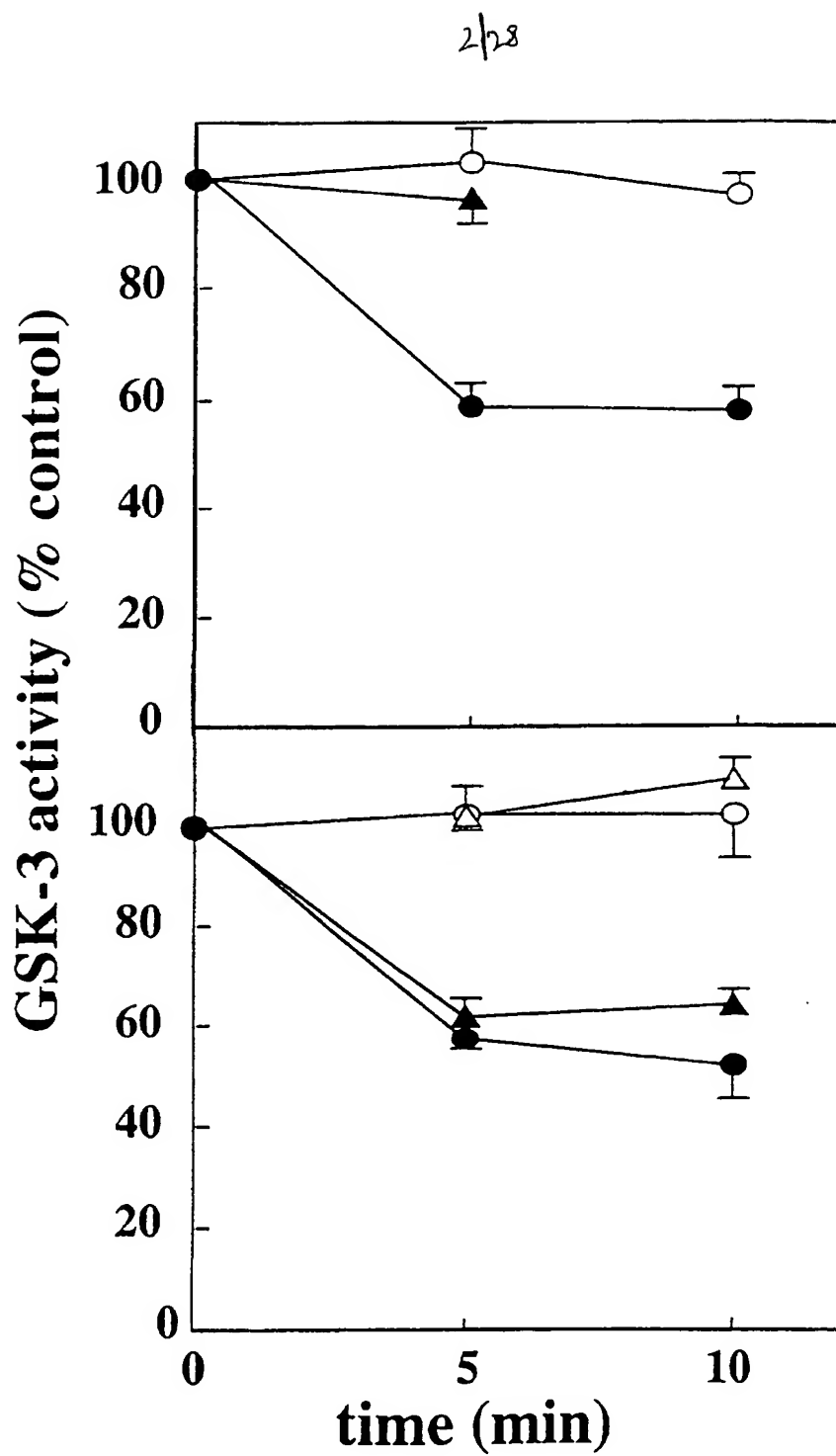


Fig. 1a





Figs. 1b &amp; 1c

3/28

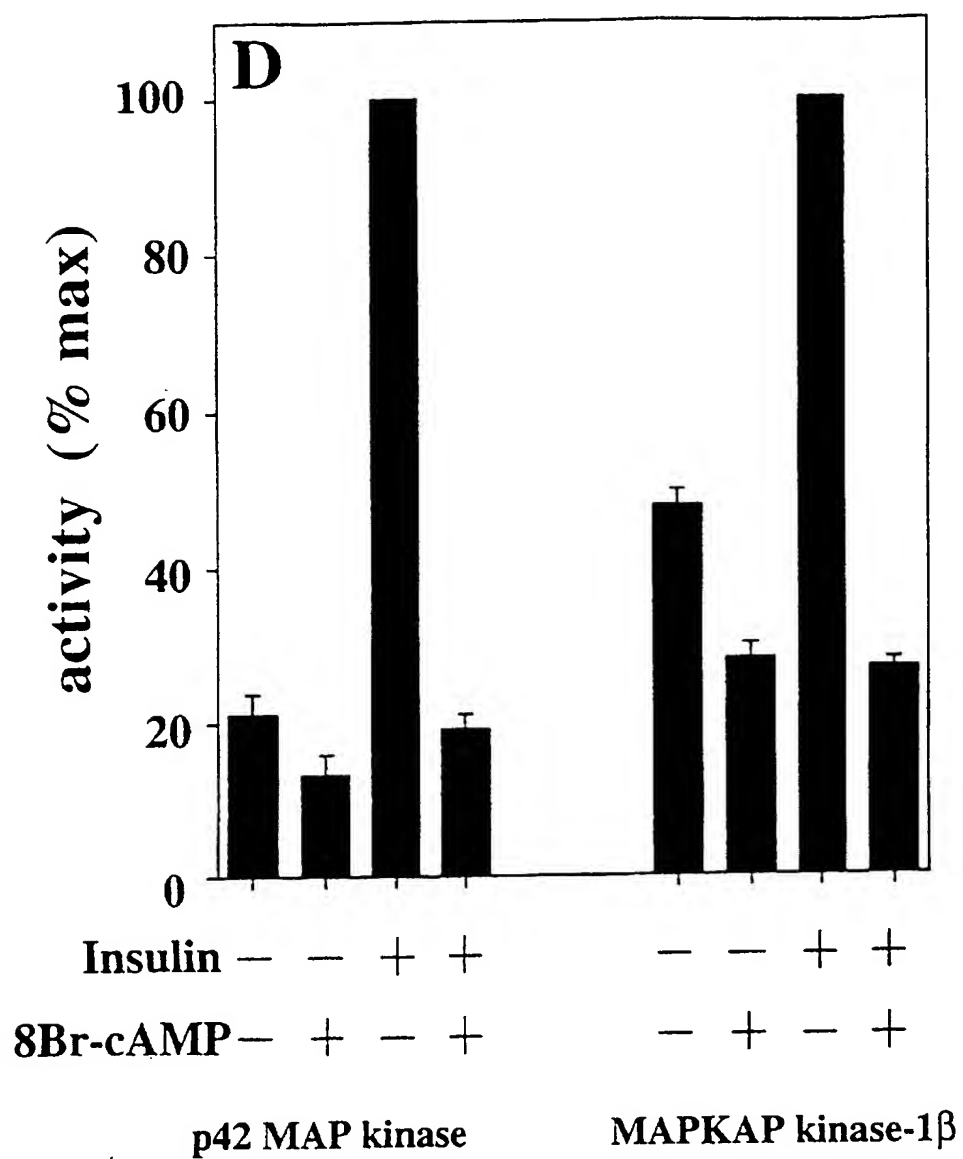


Fig. 1d

4/28

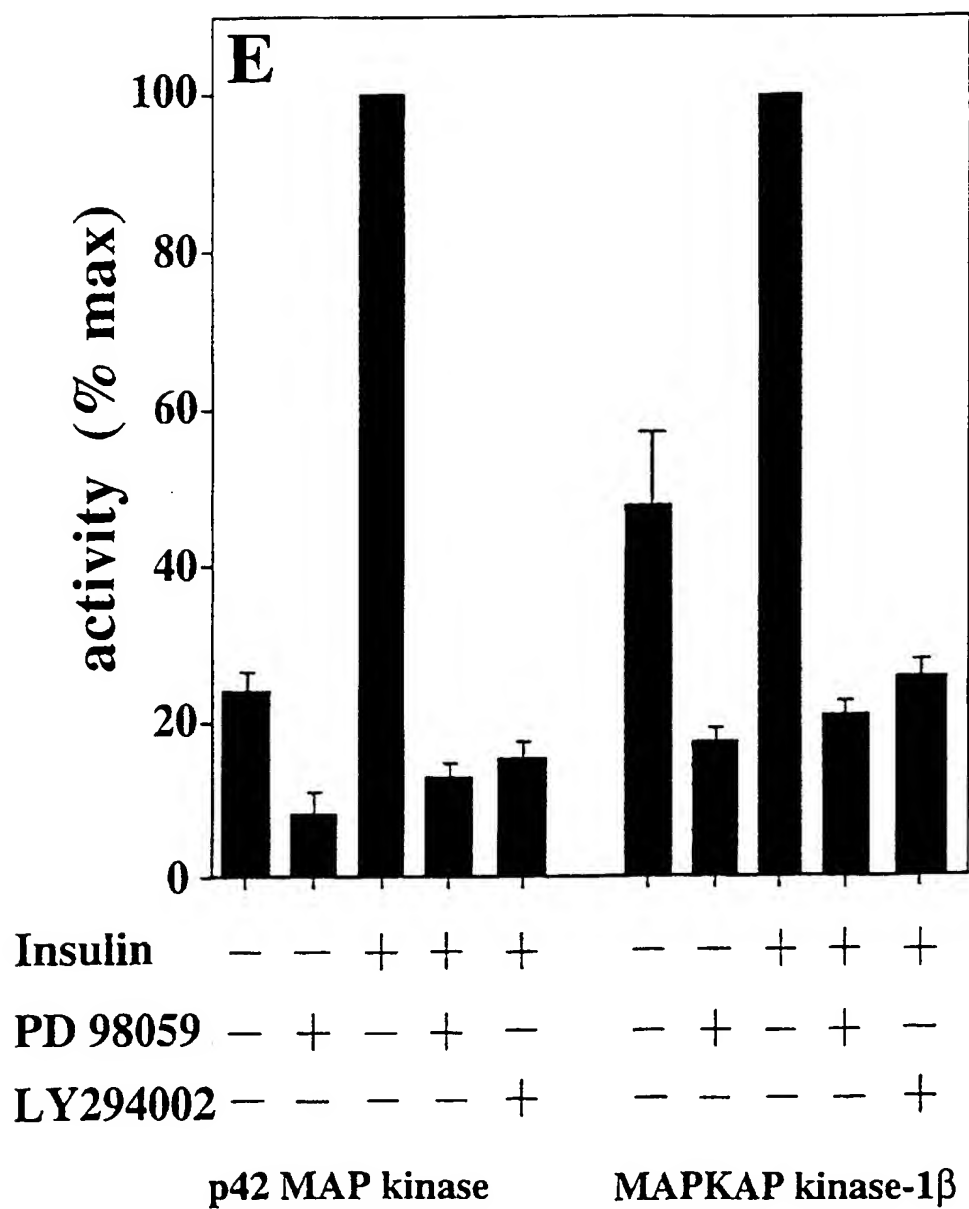


Fig. 1e

5/28

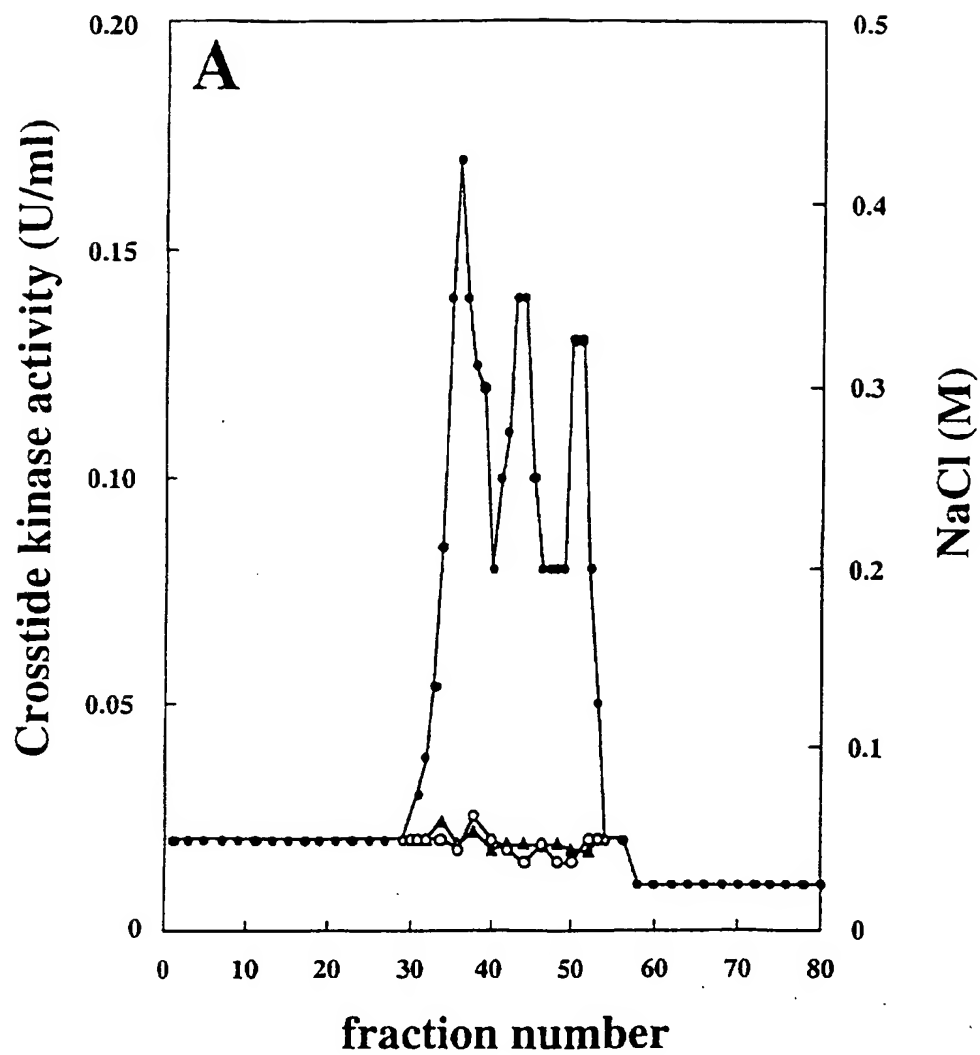


Fig. 2a

6/28

*b* $M_r$  (K) 1 2 3 4 5 6

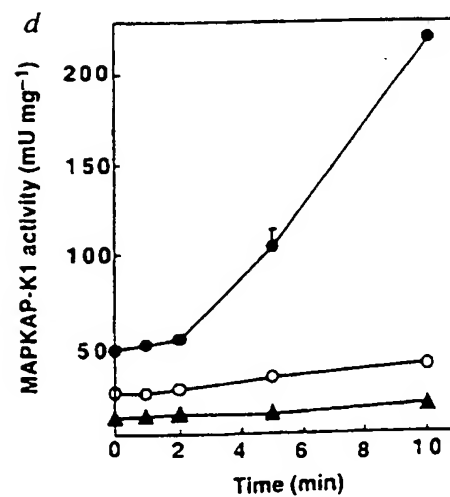
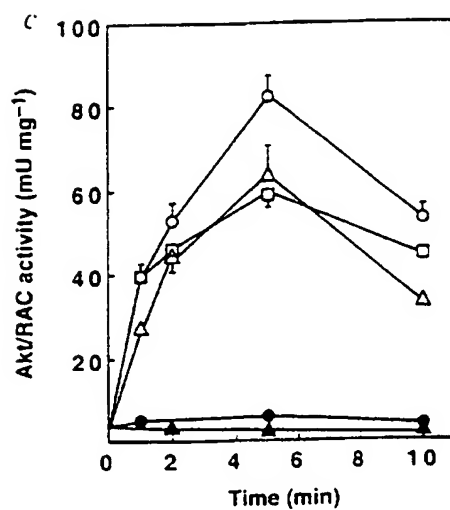
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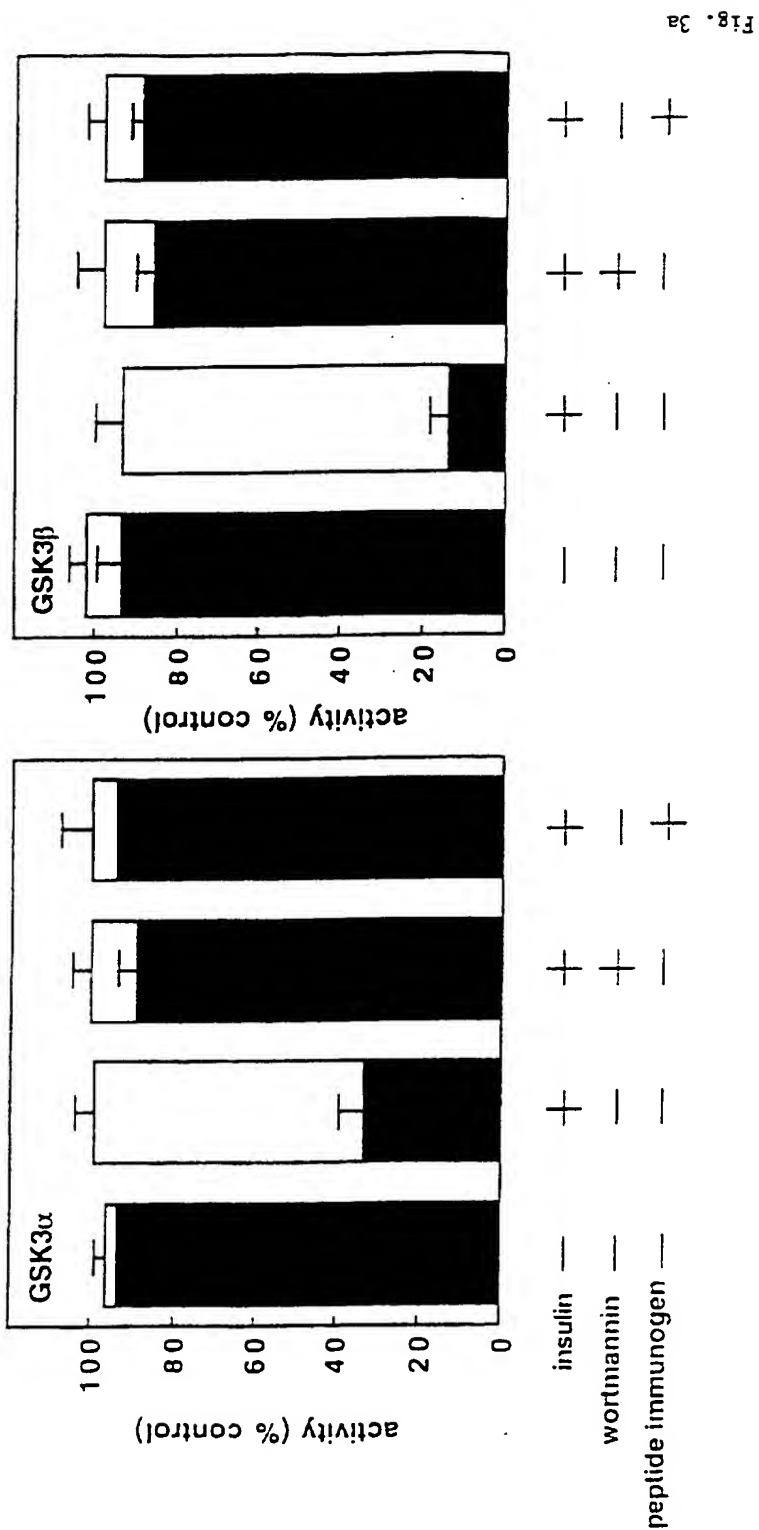
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Figs. 2b, 2c &amp; 2d

7/28



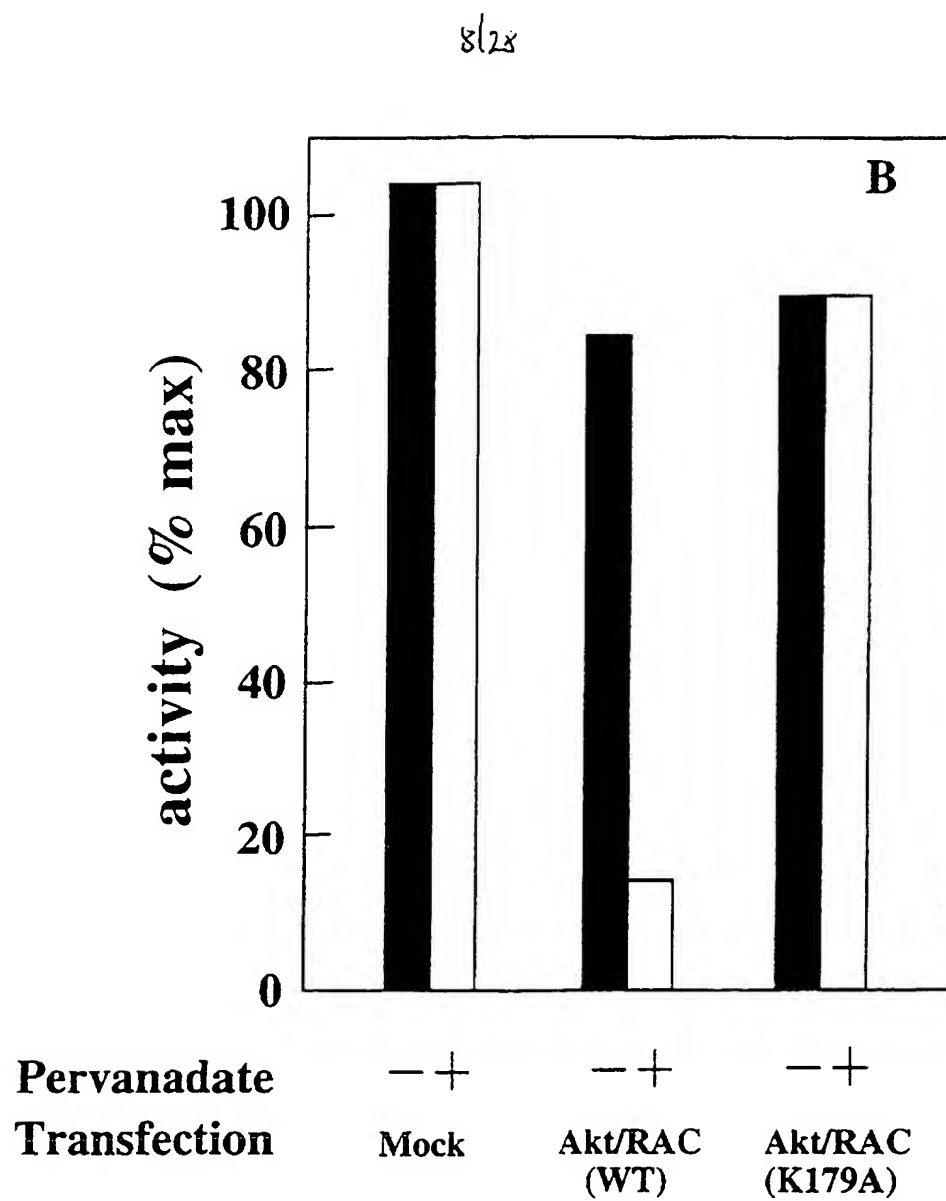
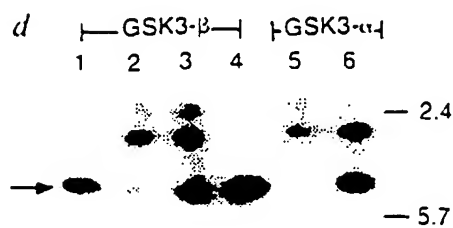
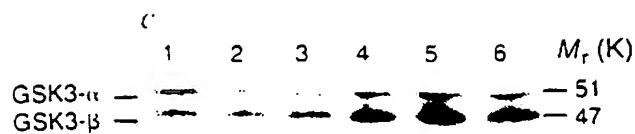
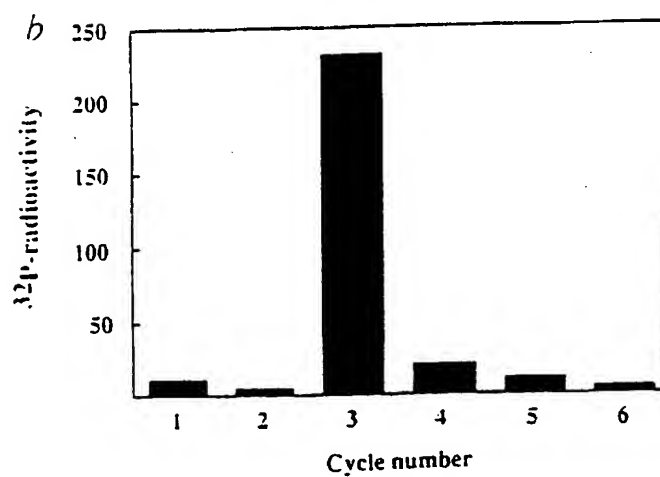
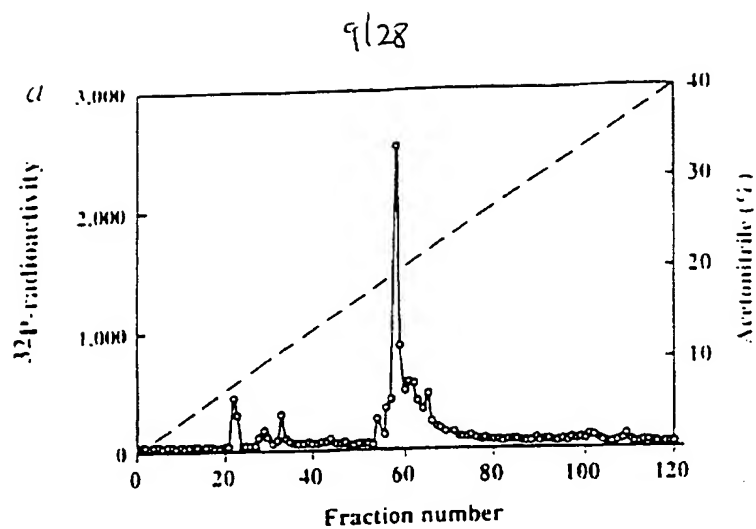


Fig. 3b

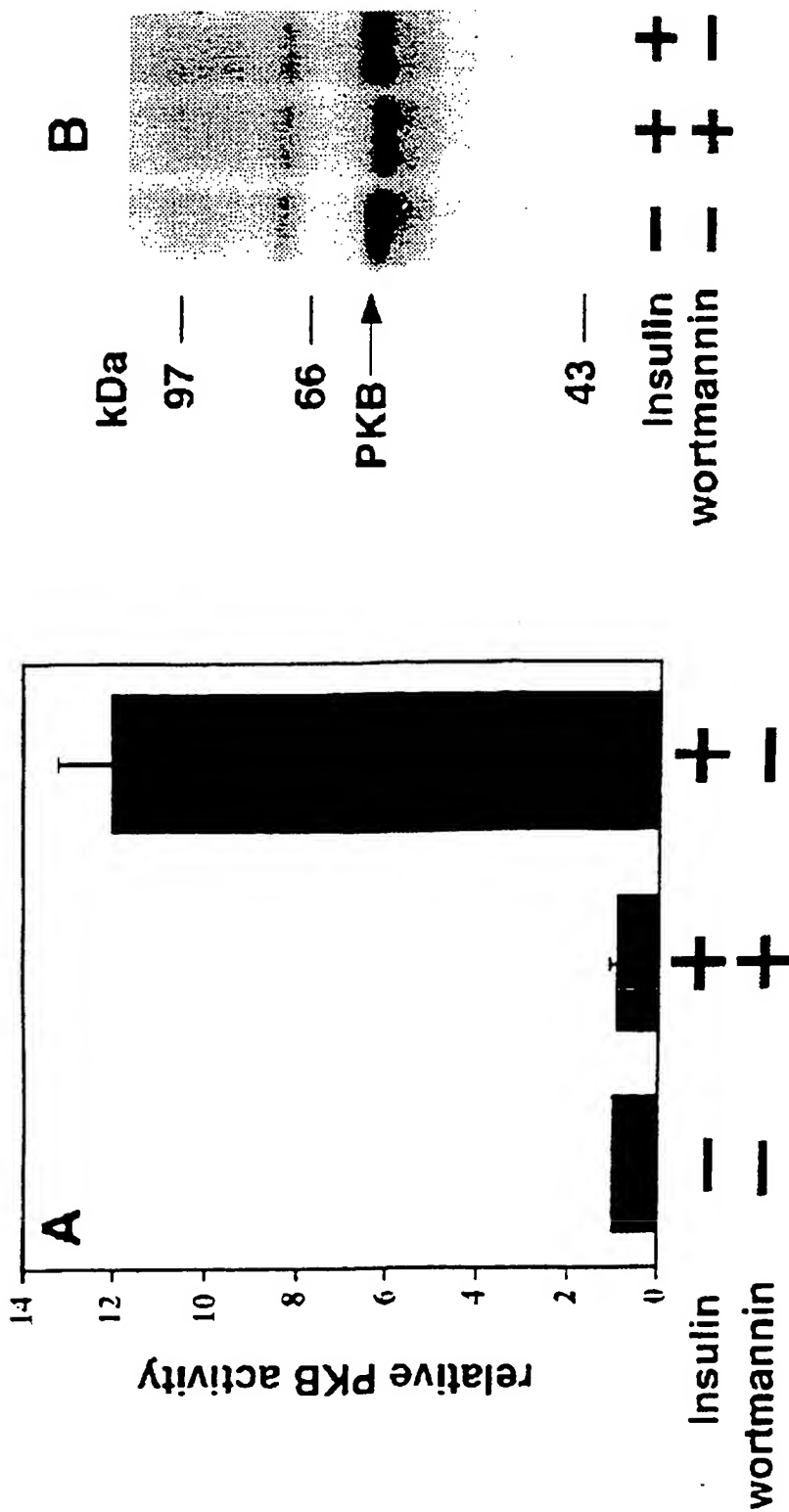


Figs. 4a, 4b, 4c &amp; 4d



10/28

Fig. 5



11/28

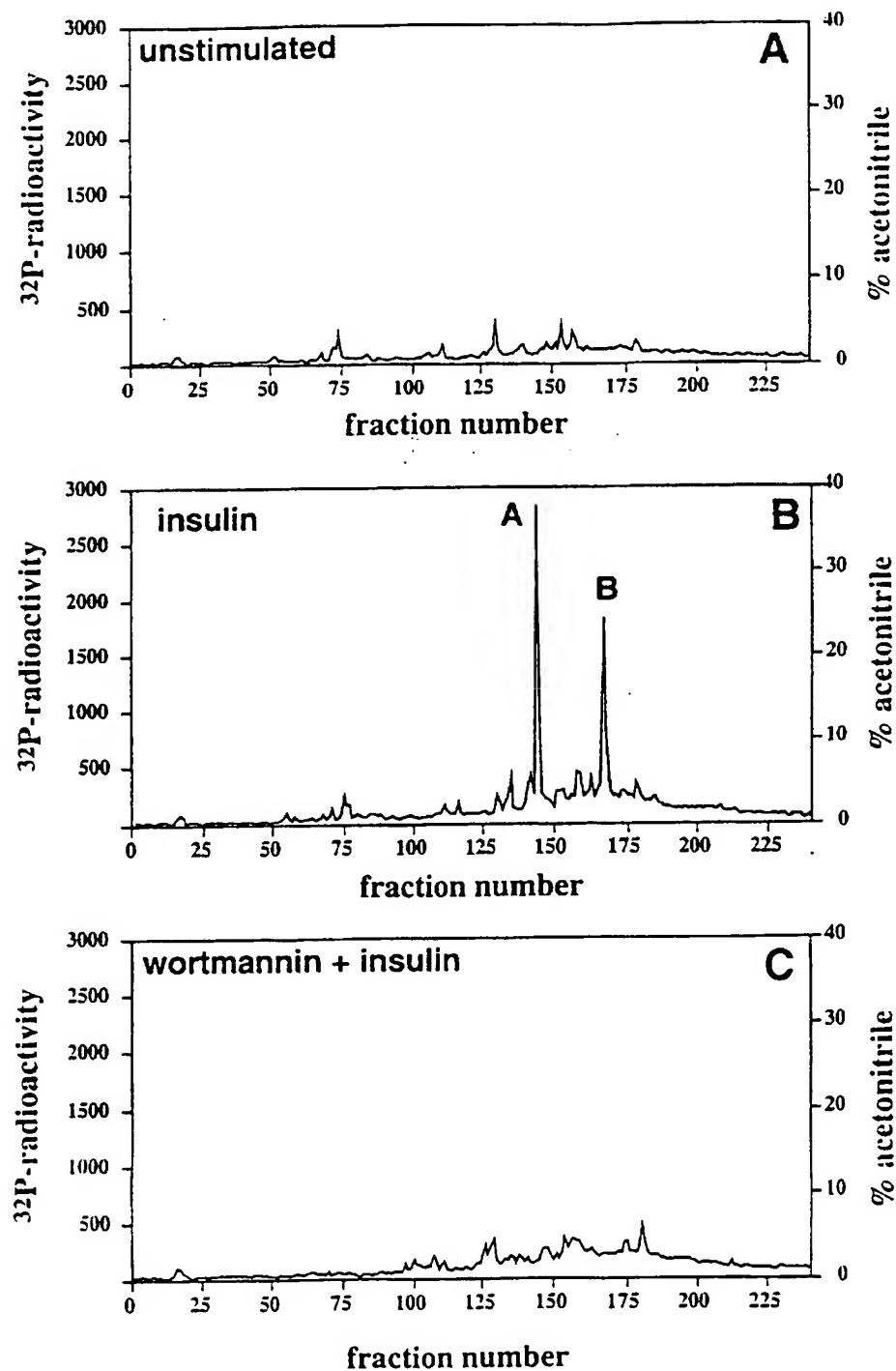


Fig. 6

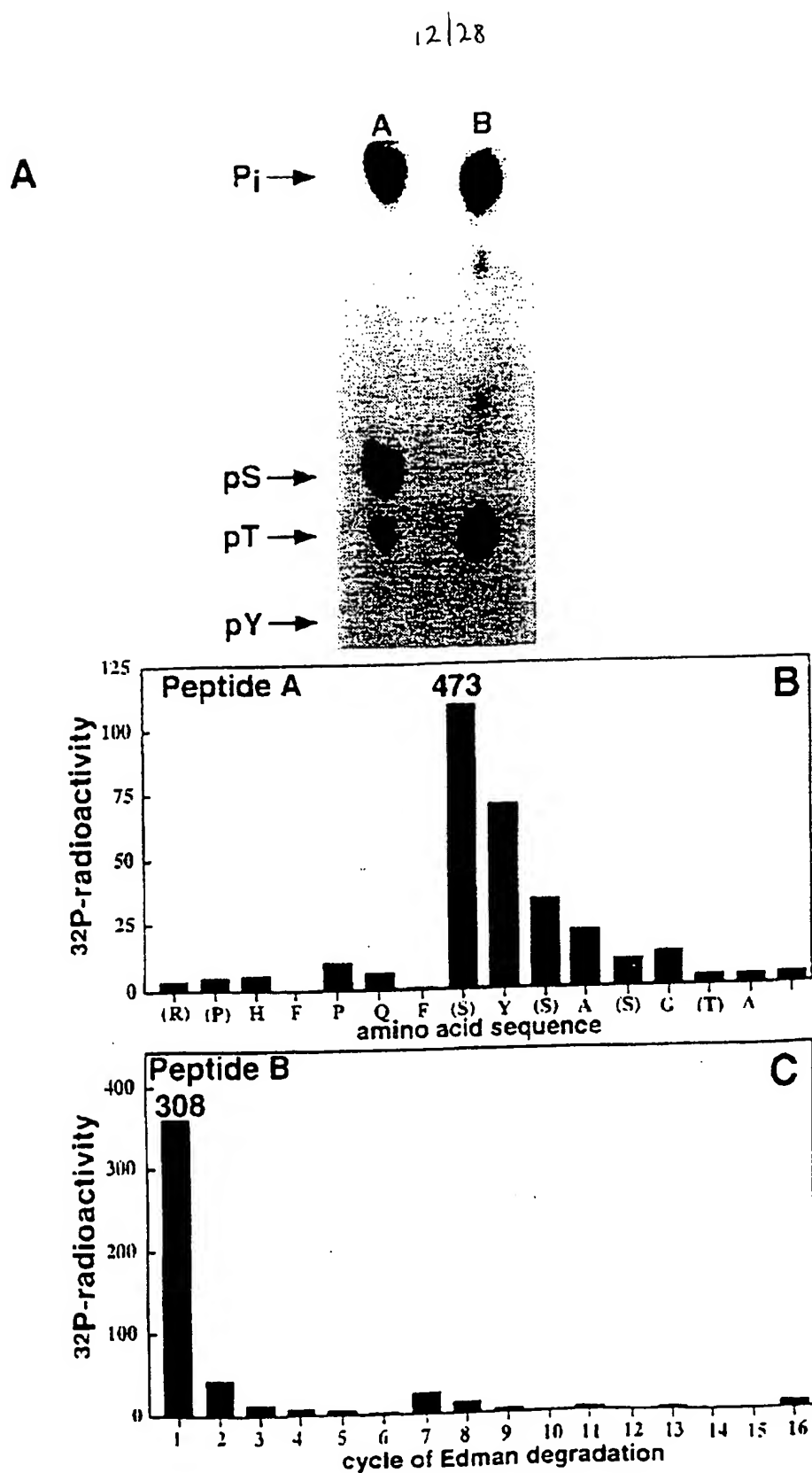


Fig. 7

13/28

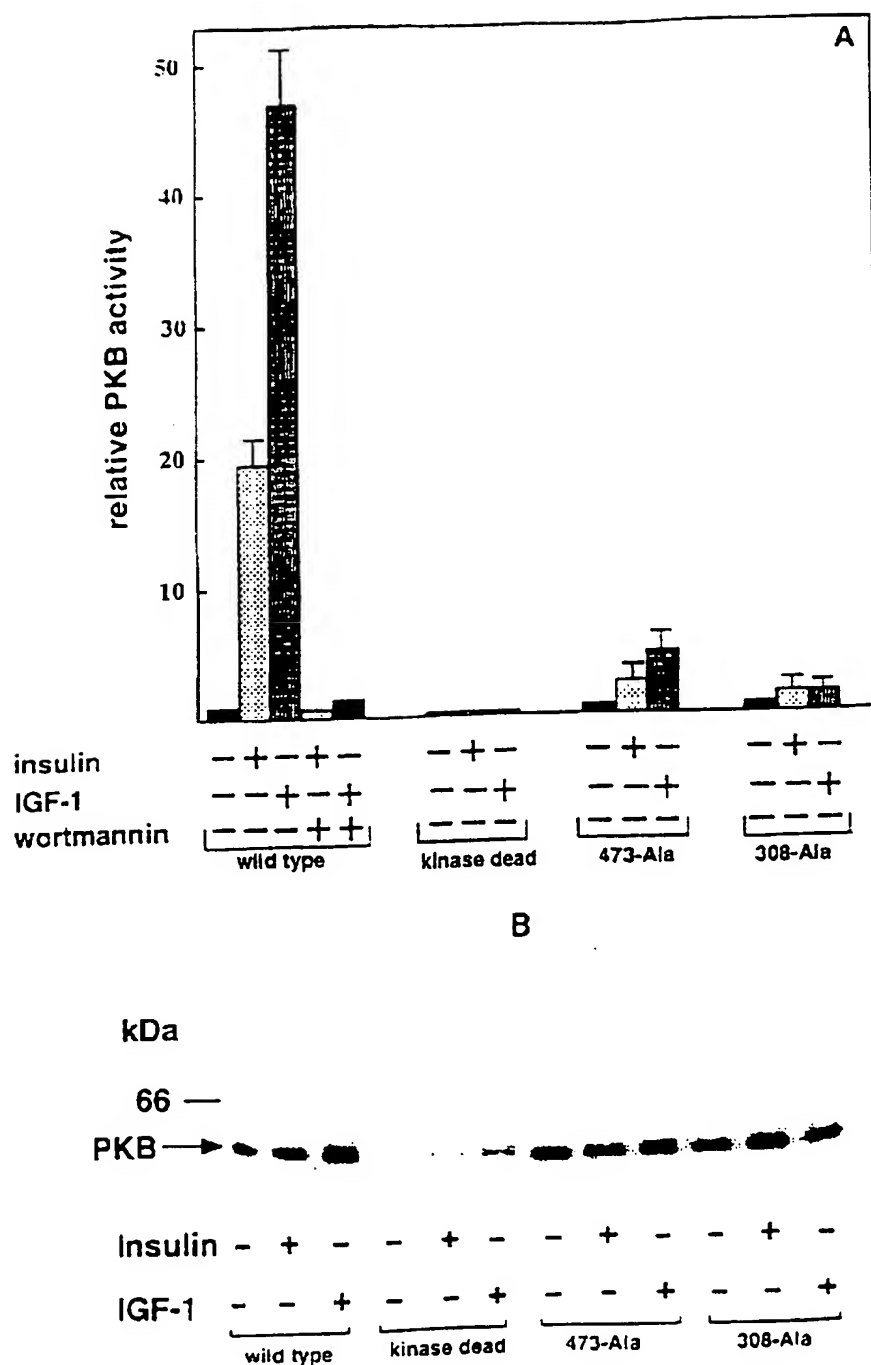


Fig. 8

14/28

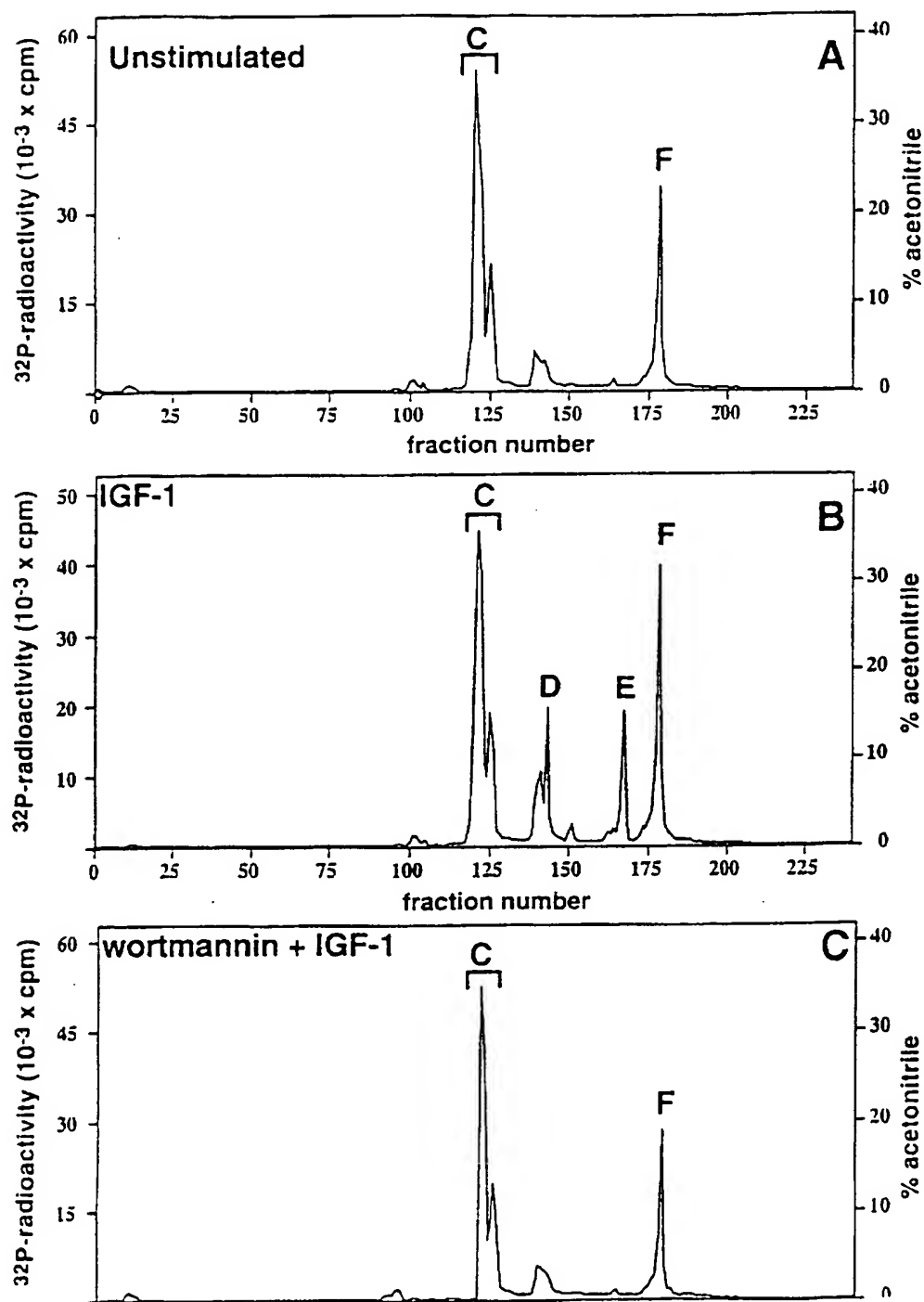
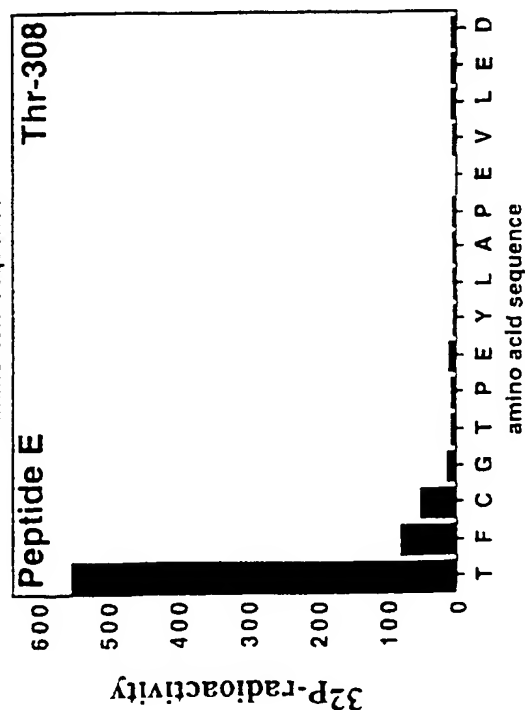
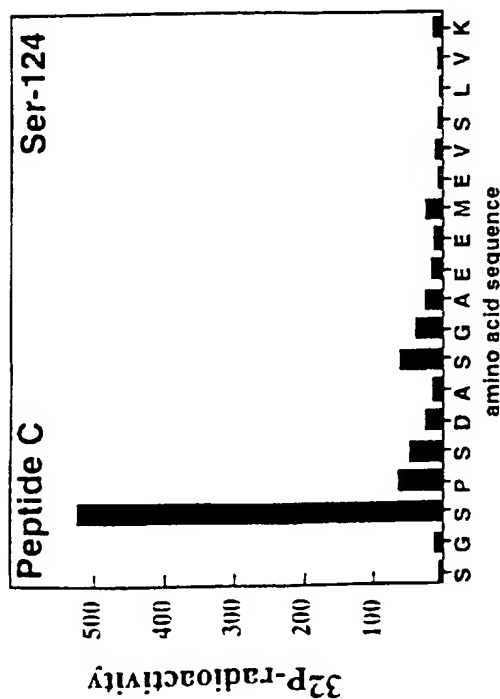
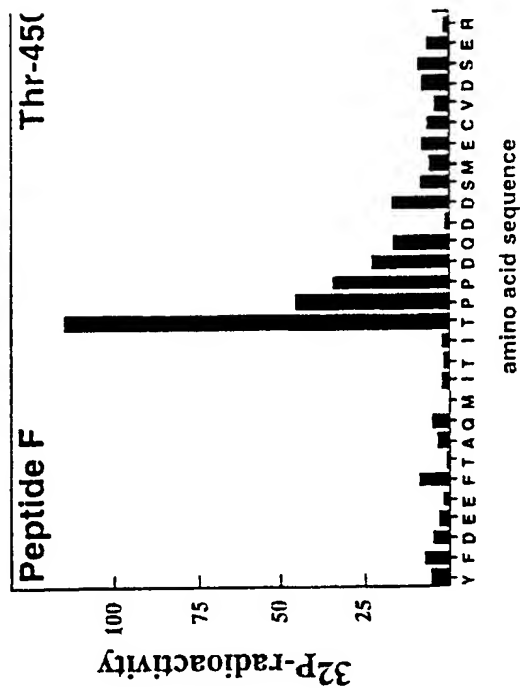
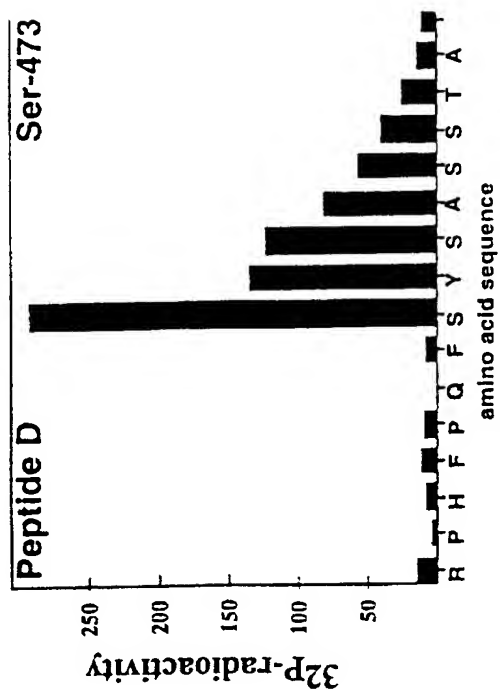


Fig. 9

15/28



16/28

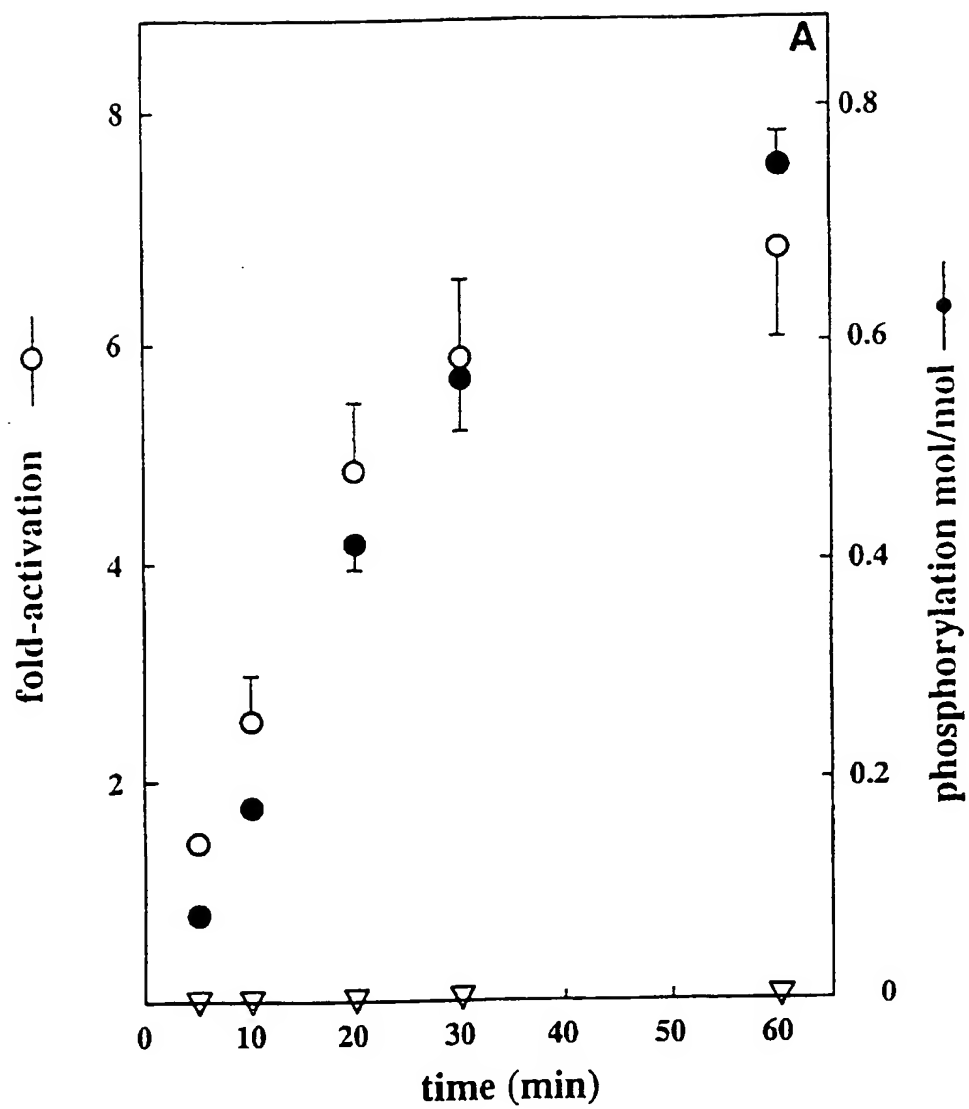
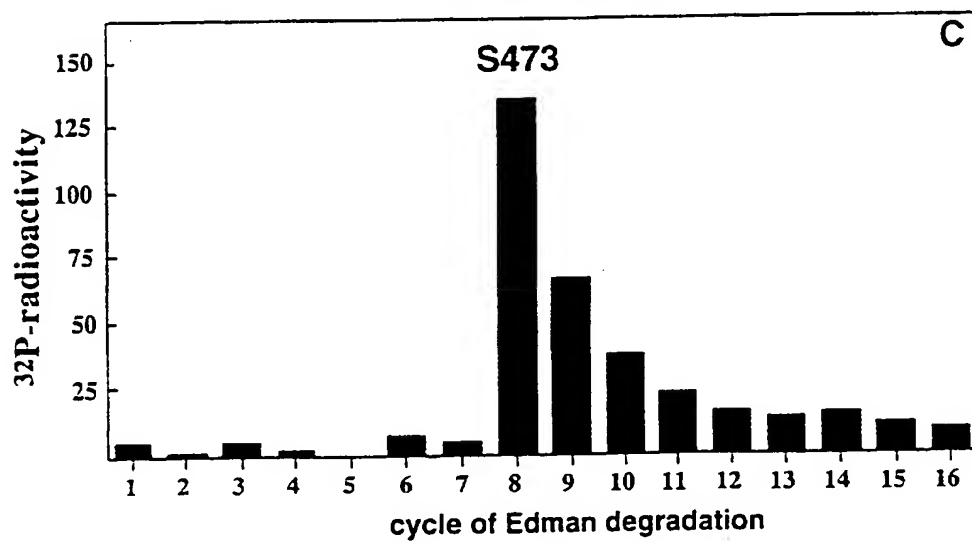
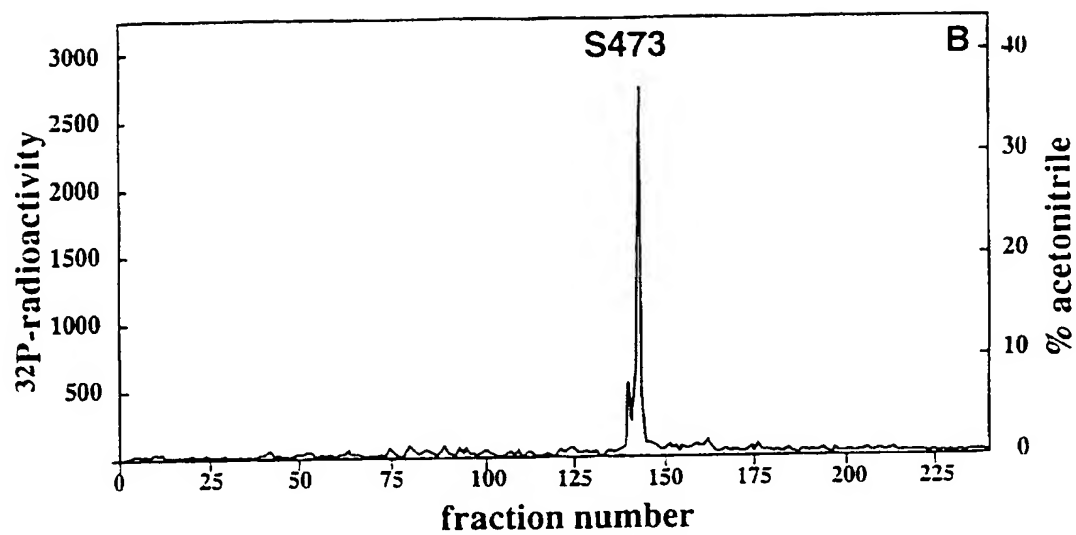


Fig. 11a

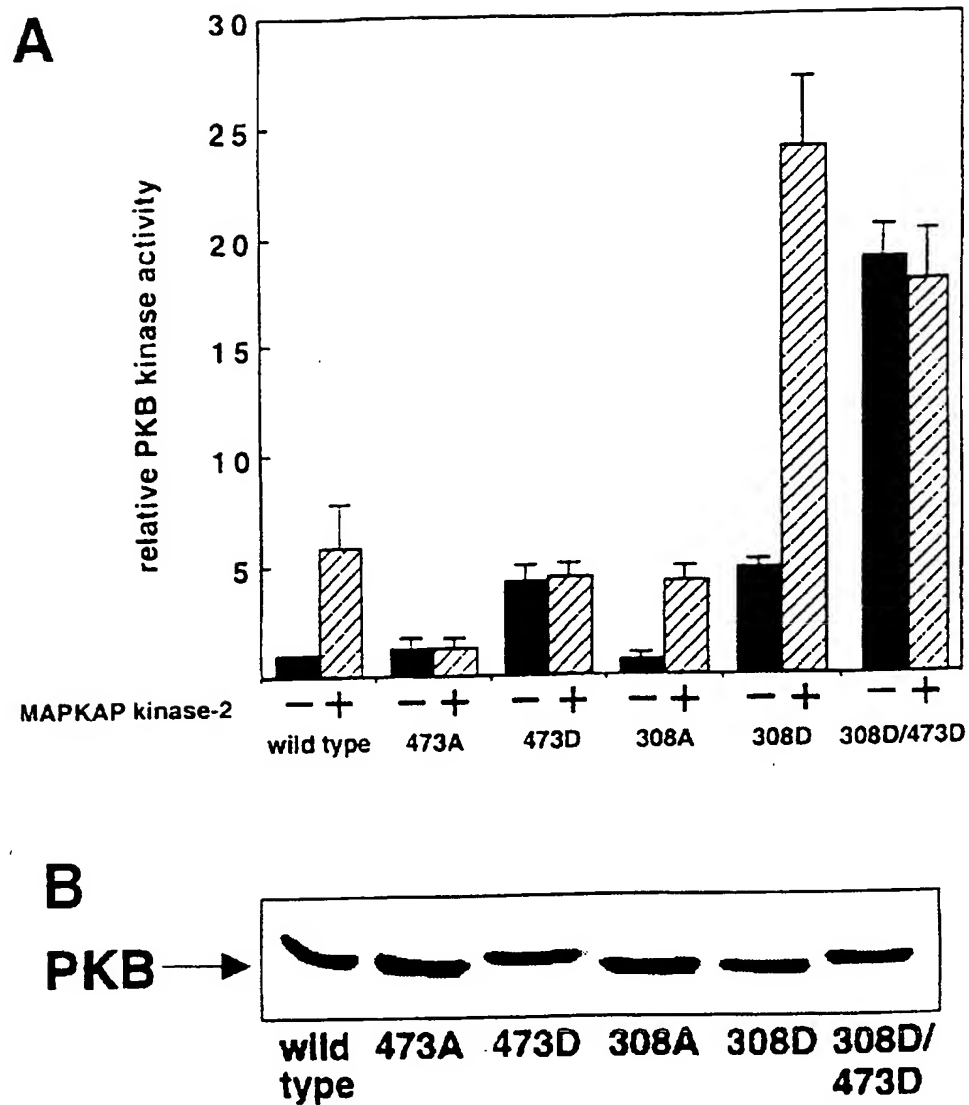
17/28



Figs. 11b &amp; 11c

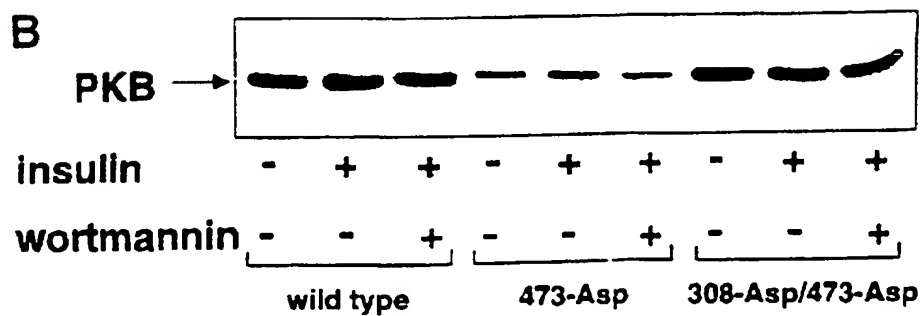
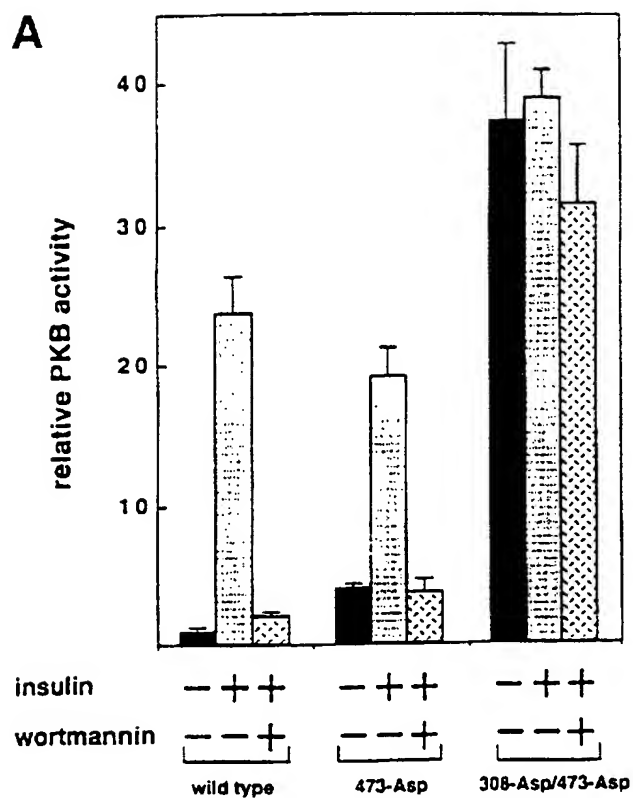


18/28



Figs. 12a &amp; 12b

19/28



Figs. 13a &amp; 13b

2c/28

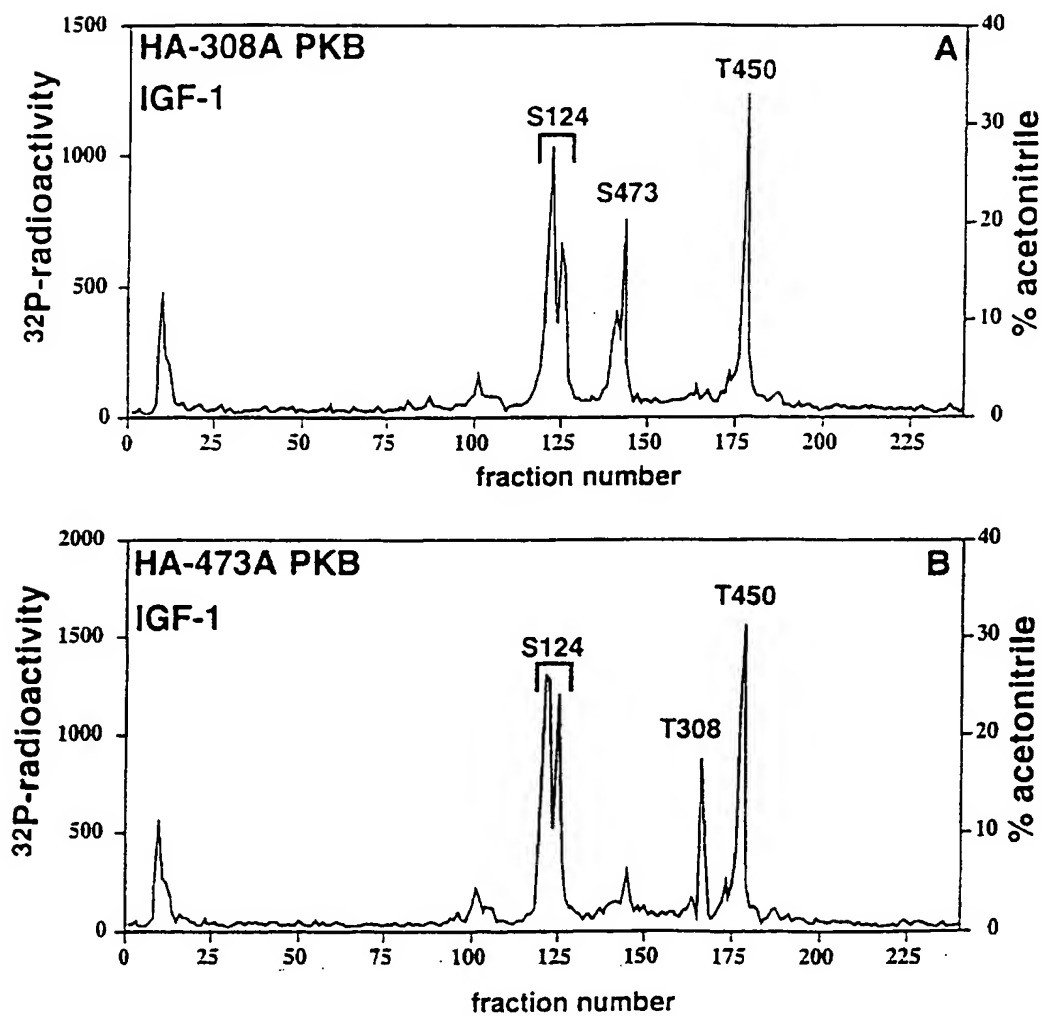


Fig. 14

2128

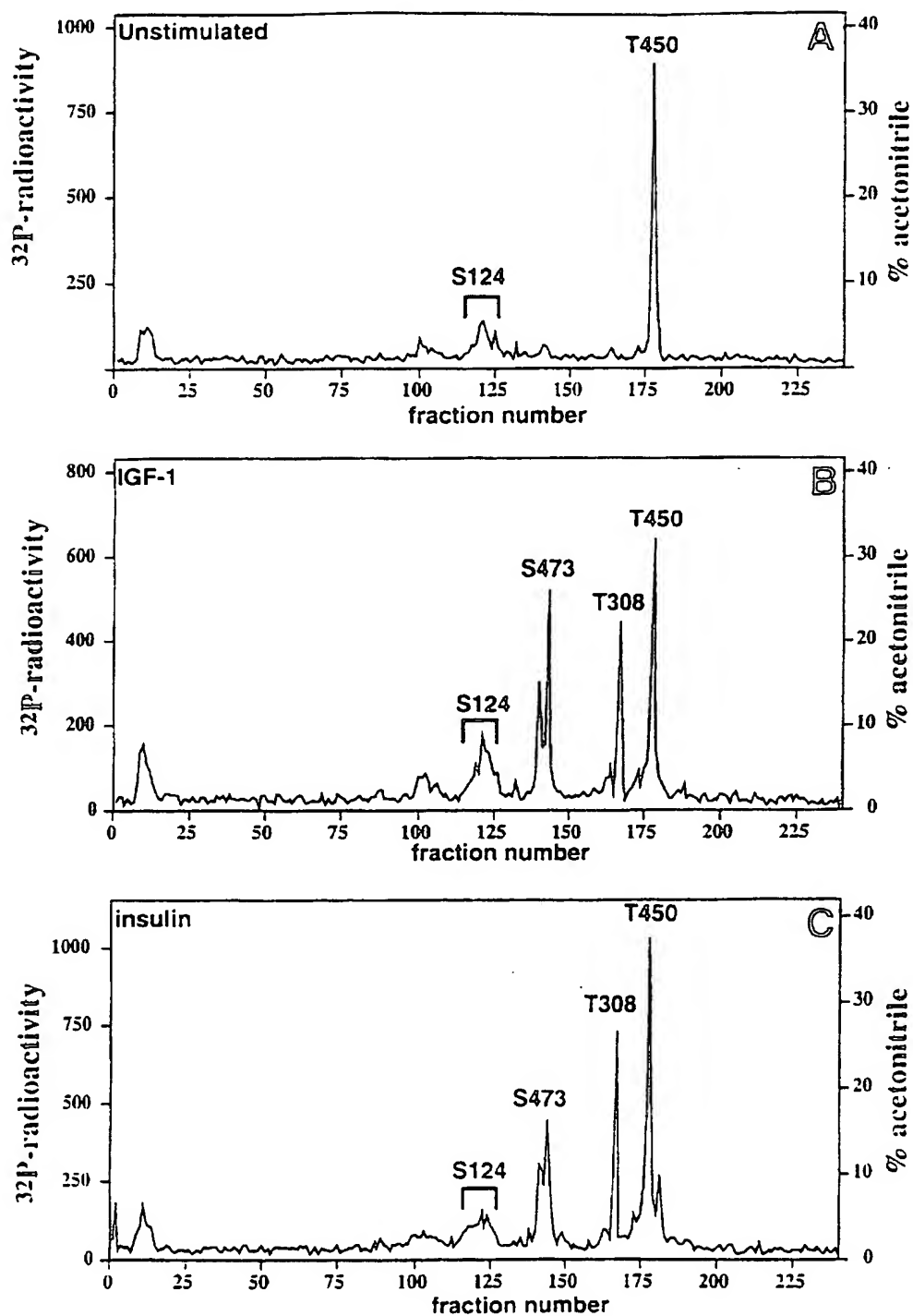


Fig. 15

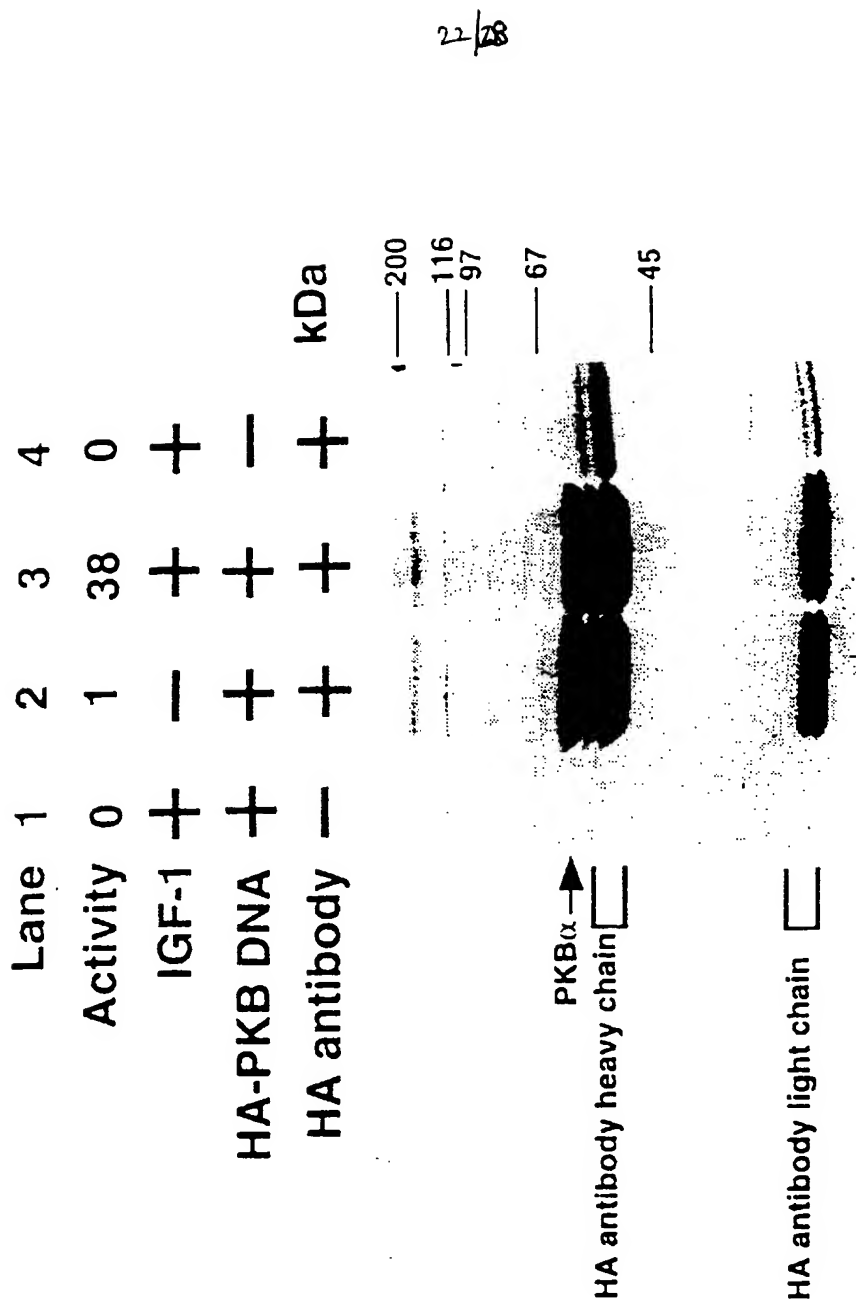


Fig. 16

23/28

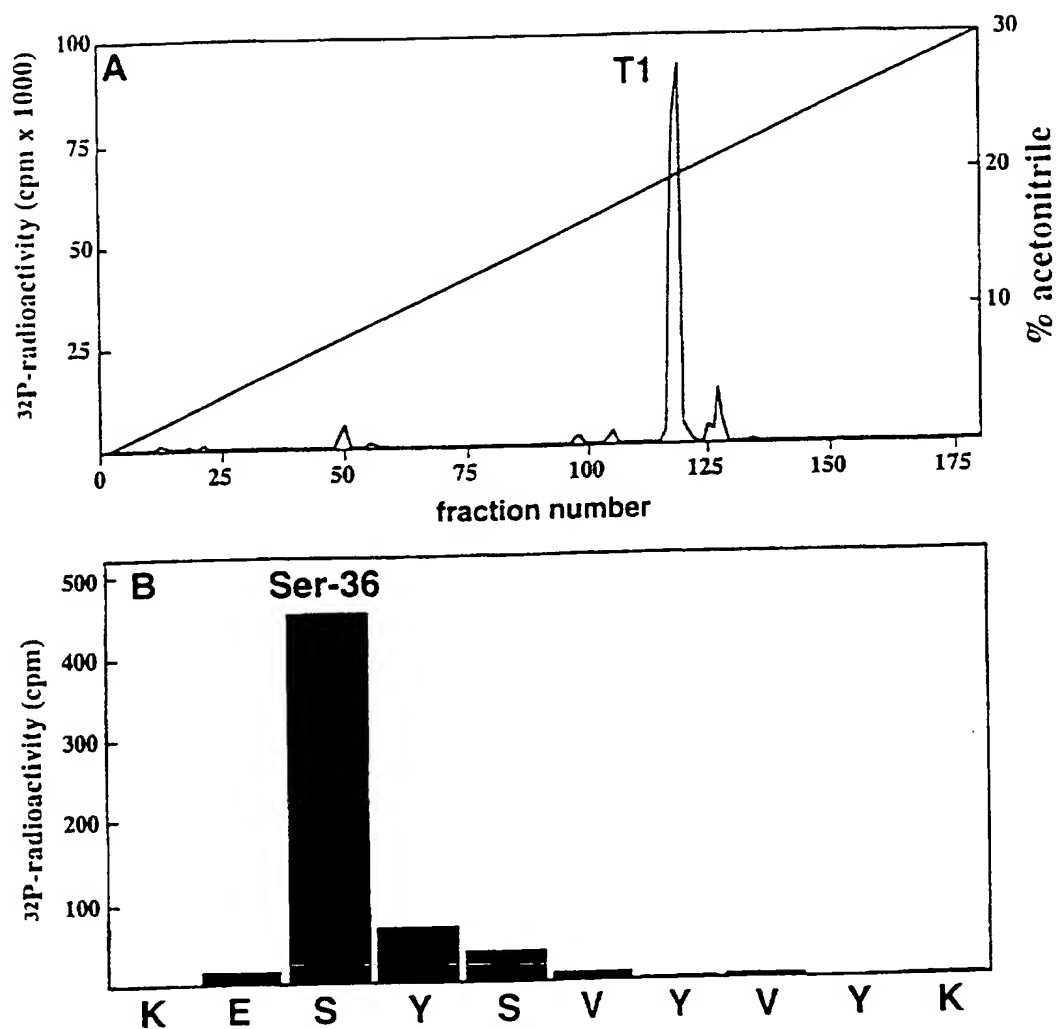


Fig. 17

24/28

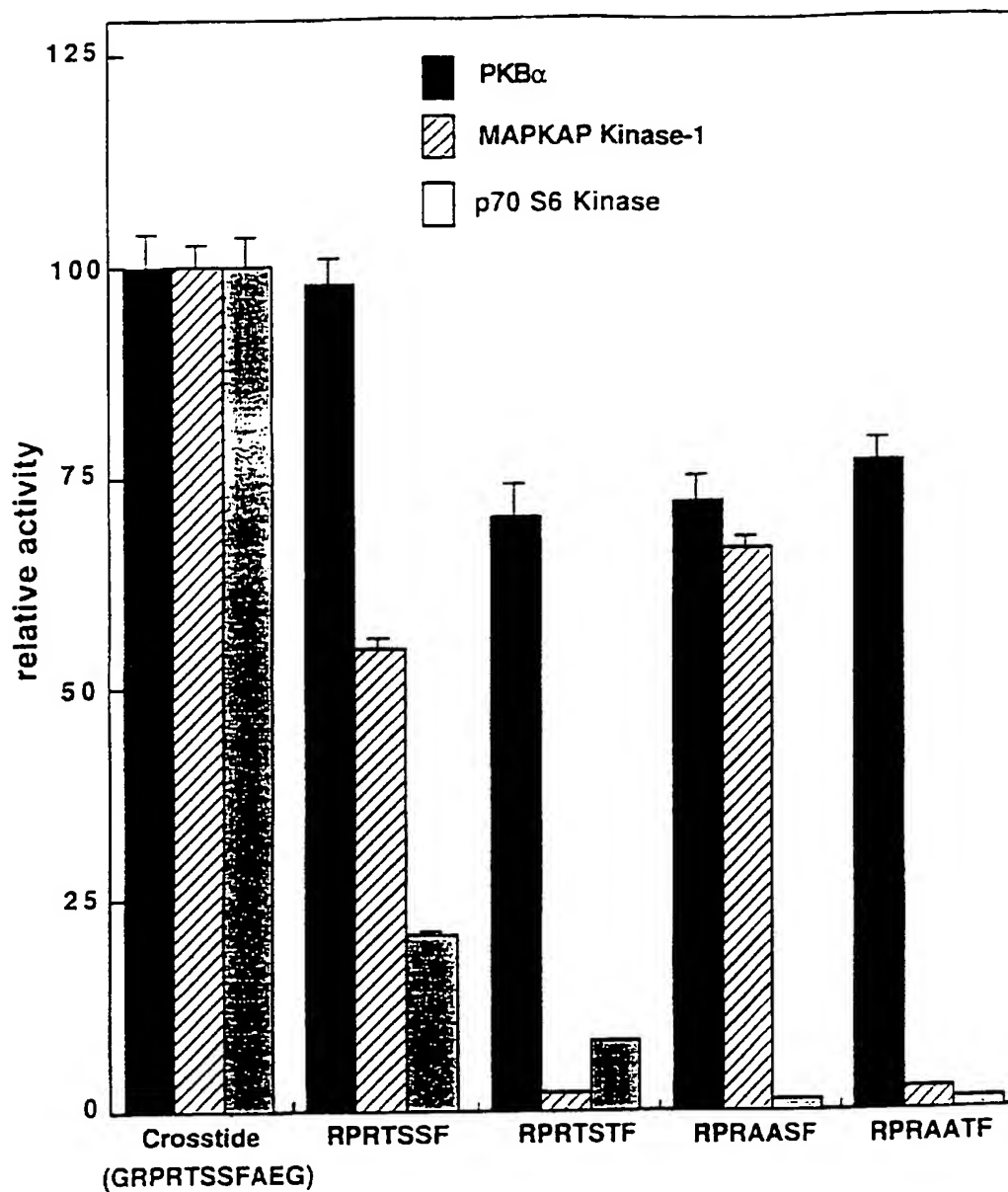


Fig. 18

25/28

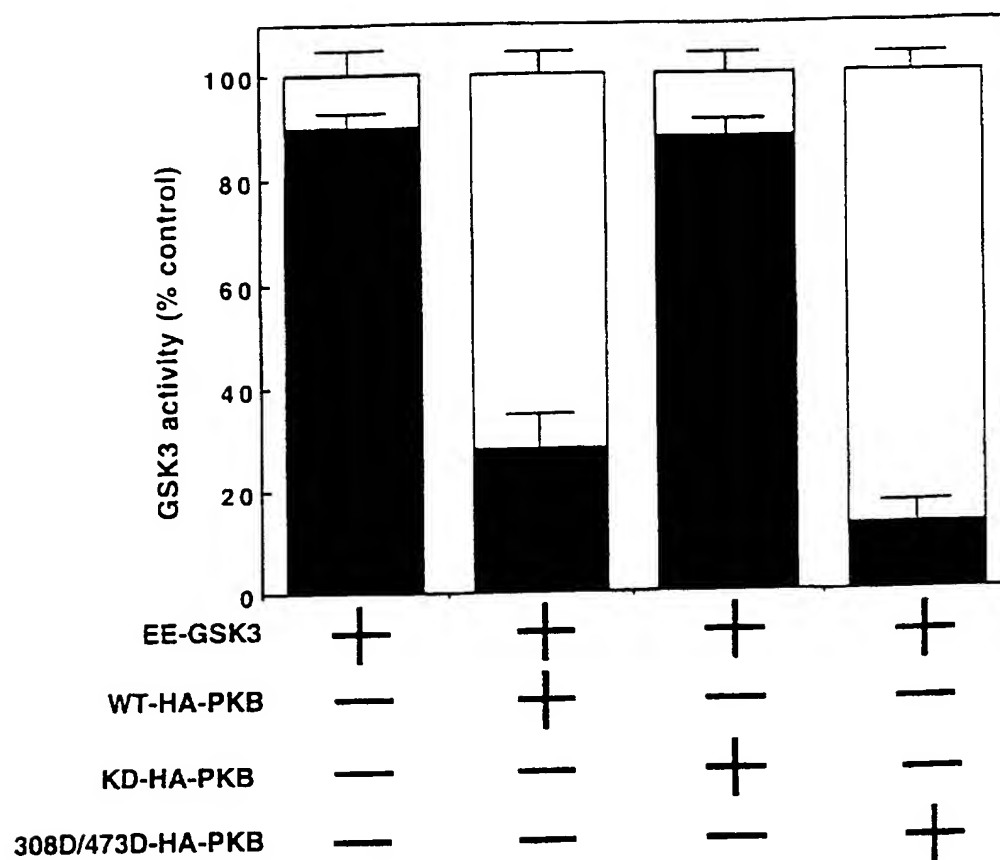


Fig. 19a



26/28

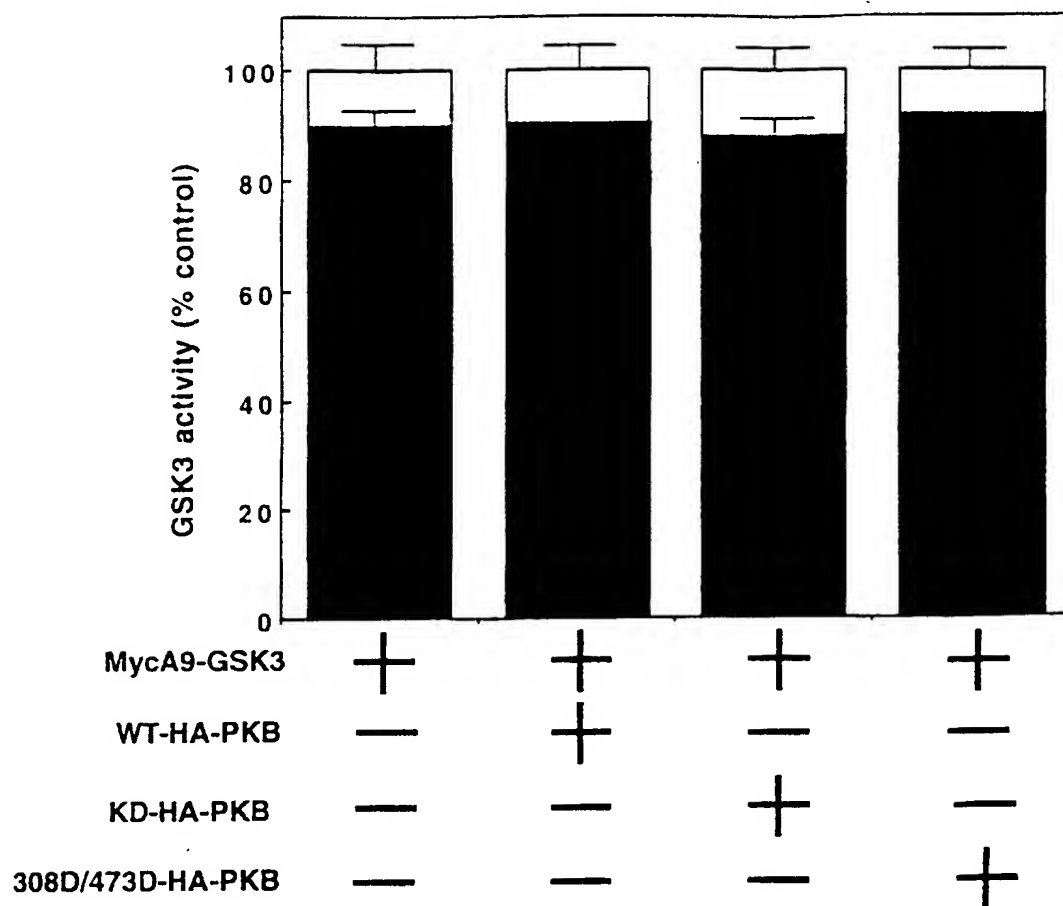


Fig. 19b

27/28

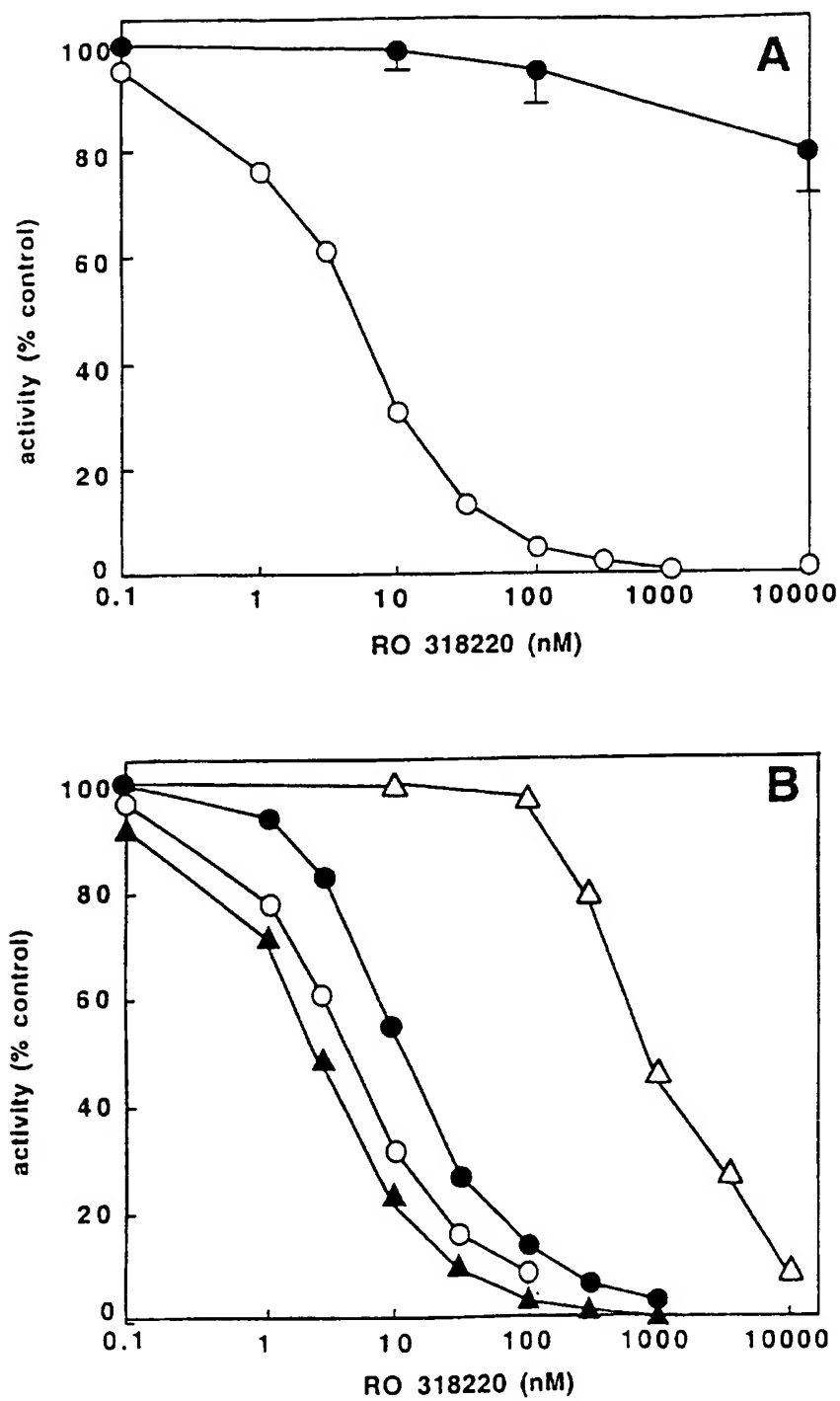


Fig. 20

28/25

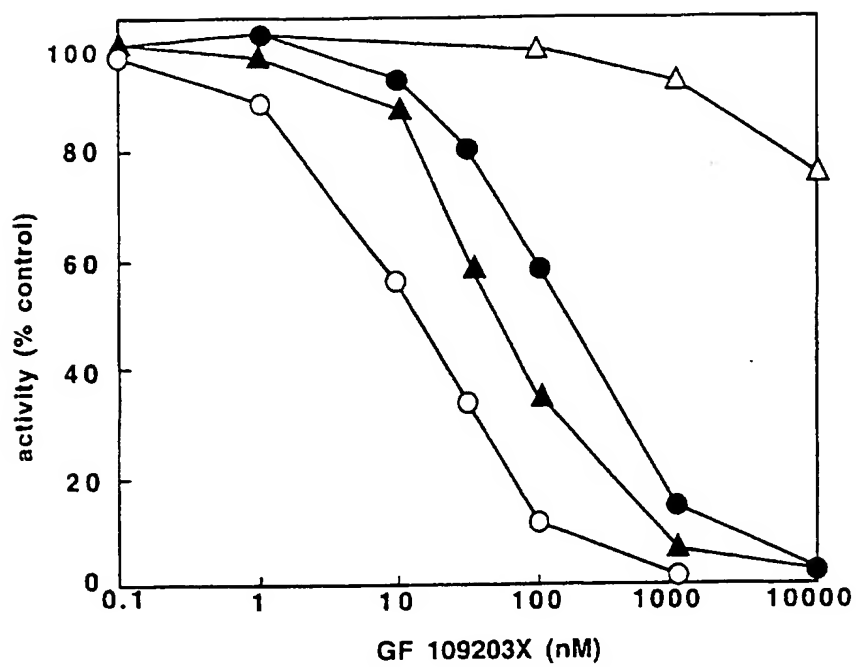


Fig. 21

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(74) Agent: MURGITROYD & COMPANY; 373 Scotland Street, Glasgow G5 8QA (GB).			
(54) Title: CONTROL OF PROTEIN SYNTHESIS, AND SCREENING METHOD FOR AGENTS			
(57) Abstract			
A method for screening for agents capable of affecting the activity of kinases GSK3 and PKB is disclosed. The method involves assessing the phosphorylation of PKB on two amino acids on the PKB molecule particularly.			

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# INTERNATIONAL SEARCH REPORT

International Application No

PCT/GB 96/03186

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A61K38/45 C12N9/12 C12Q1/48

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>NATURE, vol. 376, no. 6541, 17 August 1995, LONDON, GB, pages 599-602, XP002032104 B. BURGERING ET AL.: "Protein kinase B (c-Akt) in phosphatidylinositol-3-OH kinase signal transduction." cited in the application see abstract see page 602, left-hand column, line 48 - line 58</p> <p style="text-align: center;">--- -/--</p>	<p>1-3,5-7, 9,20,21, 23-25</p>

☒ Further documents are listed in the continuation of box C.

☐ Patent family members are listed in annex.

### \* Special categories of cited documents :

- \*A\* document defining the general state of the art which is not considered to be of particular relevance
- \*E\* earlier document but published on or after the international filing date
- \*L\* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- \*O\* document referring to an oral disclosure, use, exhibition or other means
- \*P\* document published prior to the international filing date but later than the priority date claimed

\*T\* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

\*X\* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

\*Y\* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

\*A\* document member of the same patent family

Date of the actual completion of the international search

2 June 1997

Date of mailing of the international search report

11.06.97

Name and mailing address of the ISA

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Nooij, F

## INTERNATIONAL SEARCH REPORT

Int'l Application No  
PCT/GB 96/03186

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>CELL, vol. 81, no. 5, 2 June 1995, CAMBRIDGE, MA, USA, pages 727-736, XP002032105 T. FRANKE ET AL.: "The protein kinase encoded by the Akt proto-oncogene is a target of the PDGF-activated phosphatidylinositol 3-kinase." cited in the application see abstract see page 735, left-hand column, line 2 - line 22 see figure 7 see table 1</p> <p style="text-align: center;">---</p>	<p>1-3,5-7, 9,20,21, 23-25, 27-29</p>
A	<p>NATURE, vol. 345, no. 6278, 28 June 1990, LONDON, GB, pages 825-829, XP002032106 E. SIEGFRIED ET AL.: "Putative protein kinase product of the Drosophila segment-polarity gene zeste-white3." see figure 3A</p> <p style="text-align: center;">---</p>	<p>10-13</p>
A	<p>BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 205, no. 1, 30 November 1994, DULUTH, MN, USA, pages 817-825, XP002032107 H. KONISHI ET AL.: "Molecular cloning of rat RAC protein kinase alpha and beta and their association with protein kinase C gamma." see abstract see figure 1</p> <p style="text-align: center;">---</p>	<p>10-12</p>
P,X	<p>NATURE, vol. 378, no. 6559, 21 December 1995, LONDON, GB, pages 785-789, XP002025954 D. CROSS ET AL.: "Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B." cited in the application see abstract see page 789, left-hand column, line 18 - line 33</p> <p style="text-align: center;">---</p> <p style="text-align: center;">-/--</p>	<p>1-3,5-7, 9-18,20, 21, 23-25, 27-29, 31-33</p>

# INTERNATIONAL SEARCH REPORT

International Application No  
**PL1/GB 96/03186**

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	<p>THE EMBO JOURNAL,  vol. 15, no. 23, 2 December 1996, OXFORD,  GB,  pages 6541-6551, XP002032108  D. ALESSI ET AL.: "Mechanism of  activation of protein kinase B by insulin  and IGF-1."  cited in the application  see the whole document  ---</p>	<p>1,7-9,  26-30,34</p>
P,X	<p>FEBS LETTERS,  vol. 399, no. 3, 16 December 1996,  AMSTERDAM, NL,  pages 333-338, XP002032109  D. ALESSI ET AL.: "Molecular basis for  the substrate specificity of protein  kinase B; comparison with MAPKAP kinase-1  and p70 S6 kinase."  see the whole document  -----</p>	<p>10-13</p>



# INTERNATIONAL SEARCH REPORT

International application No.

PCT/GB 96/03186

## Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 1,3-8,20-25  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 1,3-8 (all partially, as far as an in vivo method is concerned), and 20 to 25 (all completely) are directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:  
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

## Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.